DIETARY FAT AND ISCHAEMIC ARRHYTHMIAS.

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DECLARATION.

I declare that the work for this thesis was undertaken during my PhD studentship at the Cardiovascular Research Unit, Department of Medicine, University of Edinburgh and written up thereafter. I was the principal contributor to all sections except where indicated in the text.

Carol A Sargent

ABBREVIATIONS.

PUFA	Polyunsaturated fatty acid
EFA	Essential fatty acid
P/S	Polyunsaturated to saturated fatty acid ratio
GLC	Gas liquid chromatography
TLC	Thin layer chromatography
BHT	Butylated hydroxytoluene
PI	Phosphatidylinositol
PS	Phosphatidylserine
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
CL	Cardiolipin
SHR	Spontaneous hypertensive rat
VT	Ventricular tachycardia
VF	Ventricular fibrillation

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ABSTRACT.

Sudden cardiac death constitutes over a quarter of the total mortality from coronary heart disease in man. The cause of these deaths is assumed to be the cardiac rhythm disturbance, ventricular fibrillation (VF). Any intervention which might reduce VF is of major clinical importance. Diets enriched with unphysiologically large quantities of the polyunsaturated fatty acid (PUFA), 18:2(n-6) have been shown to reduce ventricular fibrillation in animals. However, such diets also result in reciprocal changes in other nutrients. The primary aim of this thesis was to confirm the antiarrhythmic effect of 18:2(n-6) on ischaemic arrhythmias using balanced semi-synthetic diets and to determine its mechanism. Arrhythmias were studied during coronary artery ligation (20 minutes) in the isolated rat heart, Langendorff perfused with a modified Krebs-Henseleit buffer (K^+=3.0mM). The influence of these diets on myocardial phospholipid fatty acid composition was also examined.

Ischaemic VF was studied in inbred rats fed a standardised semi-synthetic control diet (PUFA/saturated fatty acid ratio (P/S) 0.3) to identify an arrhythmia prone strain. The results suggested a genetic influence on the incidence of VF which was associated with altered fat metabolism.

Dietary recommendations aimed at reducing heart disease include a decrease in total fat intake to 30%

energy. The effect of different total fat diets (20, 30, 40% energy) with high or low PUFA (n-6) levels on the incidence of ischaemic VF was studied. As reported previously, an antiarrhythmic effect was observed with the high fat, high P/S ratio diet. This effect was maintained at 30% energy from fat, but at 20% energy this protective effect was attenuated. Myocardial PUFA phospholipid distribution (bar arachidonic acid) was altered with high PUFA diets. Correlations with the incidence of VF were found between dietary saturated ($r=0.825$) and polyunsaturated ($r=-0.925$) fatty acids. An unexpected pattern towards an increased number of fatty acid changes with a reduction in total fat from 40 to 30 to 20% energy was found.

Diets are enriched with PUFA's at the detriment of saturated fatty acids. To distinguish between these two factors an experiment was designed to determine the relative importance of dietary saturated fat, PUFA fat and the P/S ratio to ischaemic VF. The dominant factor was found to be the P/S ratio.

Indomethacin, a non-steroidal anti-inflammatory drug has been shown to diminish the antiarrhythmic effect of high PUFA diets. The more selective cyclo-oxygenase inhibitor flurbiprofen did not reduce the antiarrhythmic effect of high PUFA diets.

The effect of fish oil (rich in PUFA of the n-3 family) on ischaemic arrhythmias was also studied. This

was to determine whether the antiarrhythmic effect was a general PUFA property or specific to the n-6 essential PUFA's. The results from feeding realistic amounts of fish oil (0.4% kCal as (n-3) fatty acids) showed a trend towards an antiarrhythmic effect.

In conclusion, the most important antiarrhythmic dietary factor is the P/S ratio, associated with PUFA incorporation into myocardial cell membrane phospholipid. This could modulate membrane function without an alteration in prostanoid release, and thereby the propensity for ischaemic arrhythmias.

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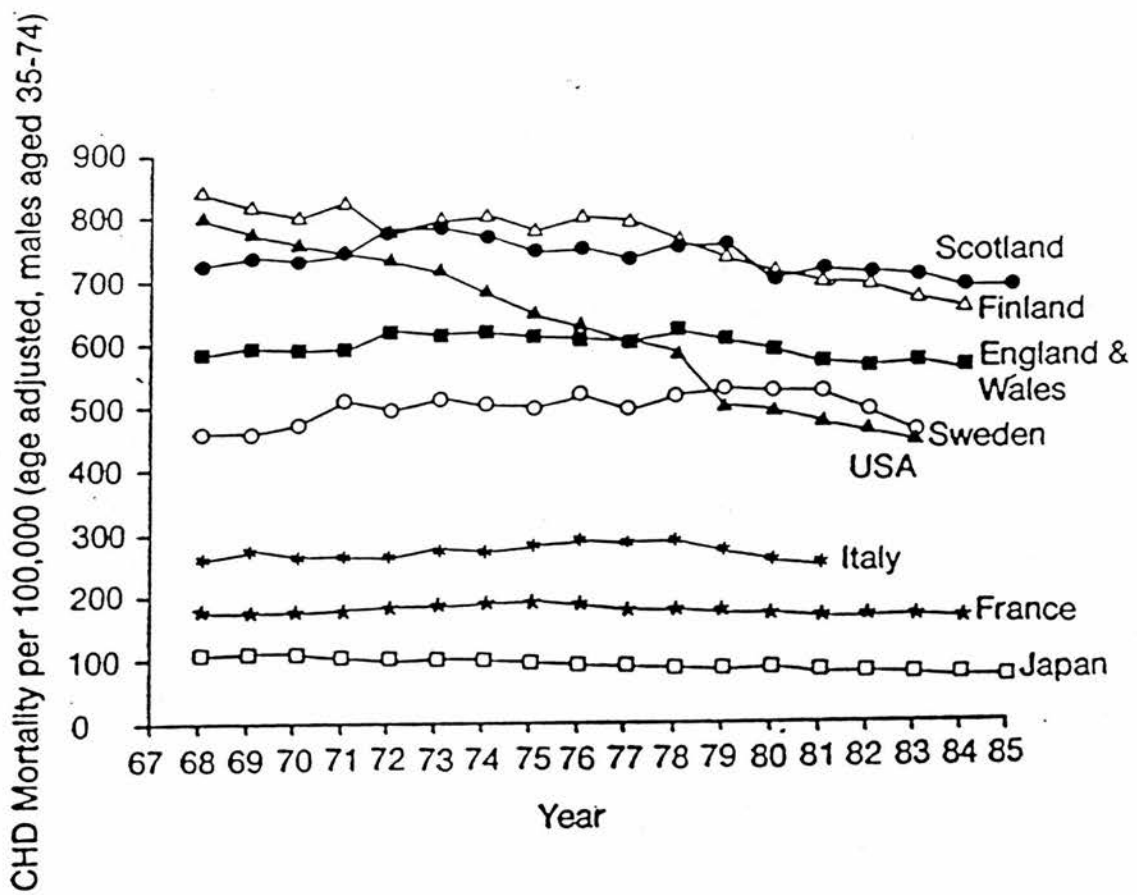
CHAPTER 1

LITERATURE REVIEW.

1.1 Introduction

Coronary heart disease is the commonest cause of death in the Western world. The precise cause of this disease is still unknown but the identification of a number of risk factors from epidemiological studies has helped to reduce mortality in some countries (Figure 1.1). However, coronary heart disease continues to be Britains greatest public health problem with death rates amongst the highest in the world [1].

FIGURE 1.1 Standardised coronary heart disease mortality rates of men aged 35-74 in several countries [2].



Understanding of coronary heart disease is complicated by geographical variation within and between countries and by the complex inter-relationships between the various risk factors. Furthermore, coronary heart disease encompasses several clinical conditions including angina pectoris, myocardial infarction and sudden cardiac death. This thesis is specifically concerned with sudden cardiac death and the role of dietary fat in the prevention of this event.

1.2 Coronary heart disease.

1.2.1 Epidemiology and identification of the risk factors of coronary heart disease.

Numerous epidemiological studies have been carried out since the end of World War II, when the mortality rate from coronary heart disease began to increase in many Western countries [3]. Only one epidemiological study has monitored sudden cardiac death alone and the bulk of the data discussed refers to total coronary heart disease mortality. Three early studies have generated most of the information about the classical risk factors of coronary heart disease;

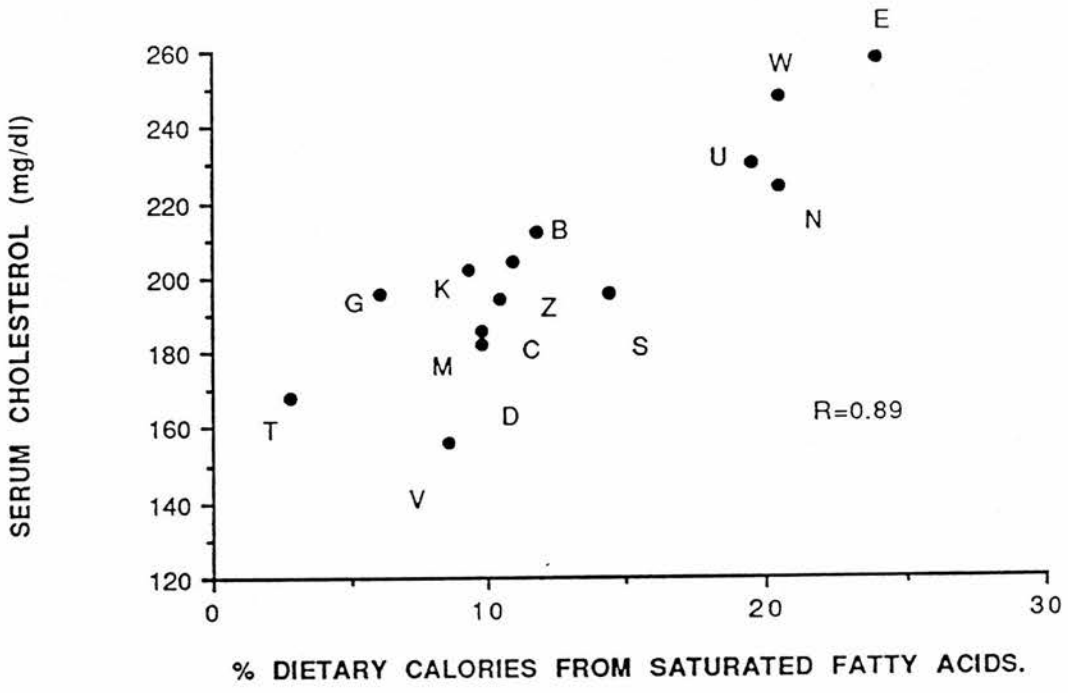
1. The International Atherosclerosis Project (IAP) [4].
2. The Seven Countries study [5].
3. The Framingham Heart Study [6].

The IAP involved investigators in 14 countries (1960-1965) in the examination of arterial specimens

(aortic, coronary, cerebral, carotid and vertebral) of men and women aged 10-69 years at autopsy using a standard protocol. The major conclusion of this study was that everyone has some degree of atherosclerosis. Accelerated conditions were primarily linked with environmental conditions and geographical location but not race or age. Furthermore the identification of many individuals with severe atherosclerosis but without clinical coronary heart disease and others with below average lesions who had demonstrated coronary heart disease indicated that atherosclerosis was not the sole determinant of coronary heart disease.

The Seven Countries study is probably the most carefully controlled study undertaken to date which assessed the risk factors for coronary heart disease in geographically and culturally distinct groups. Measurements of blood pressure, cholesterol, cigarette smoking, physical activity, age and body mass were taken from all individuals. The initial data was obtained from men aged 40-59 years (during 1957-1962) and followed up with reference to coronary heart disease at 5 and 10 years. This study identified serum total cholesterol concentration as a key factor in determining the risk of coronary heart disease. Further, total cholesterol correlated with the percentage of total energy provided by saturated fats (Figure 1.2).

FIGURE 1.2 Relationship between dietarty saturated fatty acids and serum total cholesterol comparing countries from the Seven Countries 5-year follow up.



Abbrevations B, Belgrade, Yugoslavia; C, Crevalcore, Italy; D, Dalmatia, Yugoslavia; E, East Finland; G, Corfu; K, Crete; M, Montegiorgio, Italy; N, Zutphen, The Netherlands; S, Slavonai, Yugoslavia; T, Tanushimaru, Japan; U, American railrod, USA; V, Velika Krsna, Yugoslavia; W, West Finland; Z, Zrenjanin, Yugoslavia.

Other suspected factors such as high blood pressure, cigarette smoking, lack of physical activity, age and increased body mass were stated to be relevant only when the serum total cholesterol was high. The conclusion could equally have been that all the factors including serum total cholesterol were only relevant if saturated fat intake was high. It should be noted that different countries had variations in the importance of certain risk factors and cholesterol was the only finding common to all communities. Analysis of a possible effect of dietary polyunsaturated fat on adipose tissue was not undertaken but the cohorts with the lowest mortality rates had the highest percentage of linoleic acid in their diet and vice versa for populations with the highest mortality rates.

The Framingham heart study in the USA started in 1948 and continues to the present day involving some 5000 men and women aged 30-59 years in the town of Framingham. It has provided valuable information relating possible risk factors to angina, myocardial infarction, sudden cardiac death and total coronary heart disease. This study identified elevated cholesterol levels, gender, smoking, lack of physical exercise, excess body weight and elevated blood pressure as risk factors for coronary heart disease within a population [7]. It should be noted that no information on dietary fat intake was available from this study. However, this

study did investigate individually the relationship of risk factors with myocardial infarction, angina and sudden cardiac death individually. The 20-year follow-up showed the risk factors for total coronary heart disease, myocardial infarction and angina to be similar. Sudden cardiac death lacked a relationship with elevated cholesterol but was still related to all the other risk factors, especially smoking which held a 4 fold increase in risk.

Epidemiological studies in Britain have identified similar trends in all the risk factors with the noteable exception of total serum cholesterol [8], [9]. The Edinburgh - Stockholm study [10] identified a 3-fold excess mortality from coronary heart disease, associated with low linoleic acid concentrations in adipose tissue, plasma triglycerides and plasma cholesterol esters. The involvement of dietary linoleic acid in coronary heart disease had been hypothesised very early on by the nutritionist Sinclair [11], but the data supporting this hypothesis has been inconclusive [12] [13]. The primary reason was the incorrect choice of population to study and the use of an inadequate control group. Furthermore it is diffiicult to successfully monitor dietary intake in the normal population. The Finish study overcame this problem by studying a group of institutionised mental patients, however it could be critised because this was not a normal cross-

section of the poulation. Epidemiological data has also identified a possible involvement of dietary eicosapentanoic acid from fish in the reduction of coronary heart disease [14] [15] [16], while others have found no relationship [17].

1.2.2 Sudden cardiac death.

Sudden death is poorly understood and by nature its management is difficult. Discrepancies in the classification of sudden ischaemic death contribute to the lack of understanding of this condition. For example, there are publications on "sudden death" [18] [19] and "sudden unexpected death" from the Framingham study [20]. No correlation was found between "sudden death" and serum cholesterol, but suprisingly in 1984, "sudden unexpected death" had a strong relationship with serum cholesterol ($p<0.034$). This might reflect some time-dependent process, but there was clearly a change in classification. In the earlier publications the condition was defined as "death occurring within 1 hour of symptoms" whereas in 1984 the definition was that of "death occurring within 1 hour of onset of symptoms **without** prior overt CHD and **without** other probable cause of death suggested by medical history". This discrepancy in classification renders interpretation of the results in the 1984 publication open to considerable debate. The now widely accepted definition, used

in this thesis, is that of "death due to cardiac causes within one hour of the onset of acute symptoms in an individual with or without known heart disease" [21].

The fatal event in sudden cardiac death is most commonly the onset of the lethal cardiac rhythm disturbance, (arrhythmia), ventricular fibrillation [22]. Ventricular fibrillation may accompany acute myocardial infarction [23] and/or acute myocardial ischaemia [24] precipitated by coronary artery thrombus formation [25] or spasm [26] against the background of coronary heart disease. Significantly the common post-mortem finding in cases of sudden ischaemic death is that of severe coronary atherosclerosis [27]. However, not all patients with known atherosclerosis die suddenly and individuals with electrolyte imbalance [28], drug intoxication [29] or congenital electrophysiological abnormalities (eg long QT syndrome [30]) can also develop ventricular fibrillation.

The identification of specific risk factors in sudden cardiac death is poorly documented because of the non-uniformity of classification explained above. However, from the data available, the risk factors predicting sudden cardiac death over the course of decades (20-26 years) do appear to be similar to those for coronary heart disease [31]. This is not suprising as statistically 90% of all sudden deaths occur in individuals with existing coronary heart disease [32].

1.2.3 Prevention of sudden cardiac death.

The previous section illustrated the lack of basic knowledge of the factors leading to sudden cardiac death. The early identification of those individuals at particular risk of arrhythmias is rarely successful and the treatments available for this population are limited. Improvements in identification may come with prolonged holter ECG monitoring [33] and new developments such as body surface mapping [34] and/or high fidelity ECG recordings [35].

VF can be readily reversed by the D.C. discharge from an electronic defibrillator, however such equipment is not readily available in the community. The introduction of the surgically implantable defibrillator has been shown to be effective in the prevention of sudden cardiac death [36]. But, the effect of frequent defibrillation of the heart may be detrimental and use is currently restricted to patients who have already demonstrated a serious arrhythmia and survived.

Primary prevention strategies aimed at a reduction in the classical risk factors for CHD; smoking, cholesterol, hypertension, etc have had limited success as evidenced by the decline in coronary heart disease mortality in the United States with no change in the fraction of sudden deaths [19].

Conventionally drug treatment is only introduced after an arrhythmic event. The Class 1 antiarrhythmic

rugs are the most common agents prescribed to individuals who survive such an episode. However, these drugs have side effects including bradycardia, hypotension, gastrointestinal upset and allergic reactions [37] and can also prove to be pro-arrhythmic. Indeed a recent attempt to assess the benifits of the prophylactic antiarrhythmic drug treatment, flecainide, in a selected population produced the disturbing finding of increased mortality in one of the treated groups [38] giving rise to considerable concern.

In conclusion, there is no factor which has been identified which is effective in primary prevention and the treatment available to prevent recurrence is less than ideal.

1.2.4 Diet and sudden cardiac death.

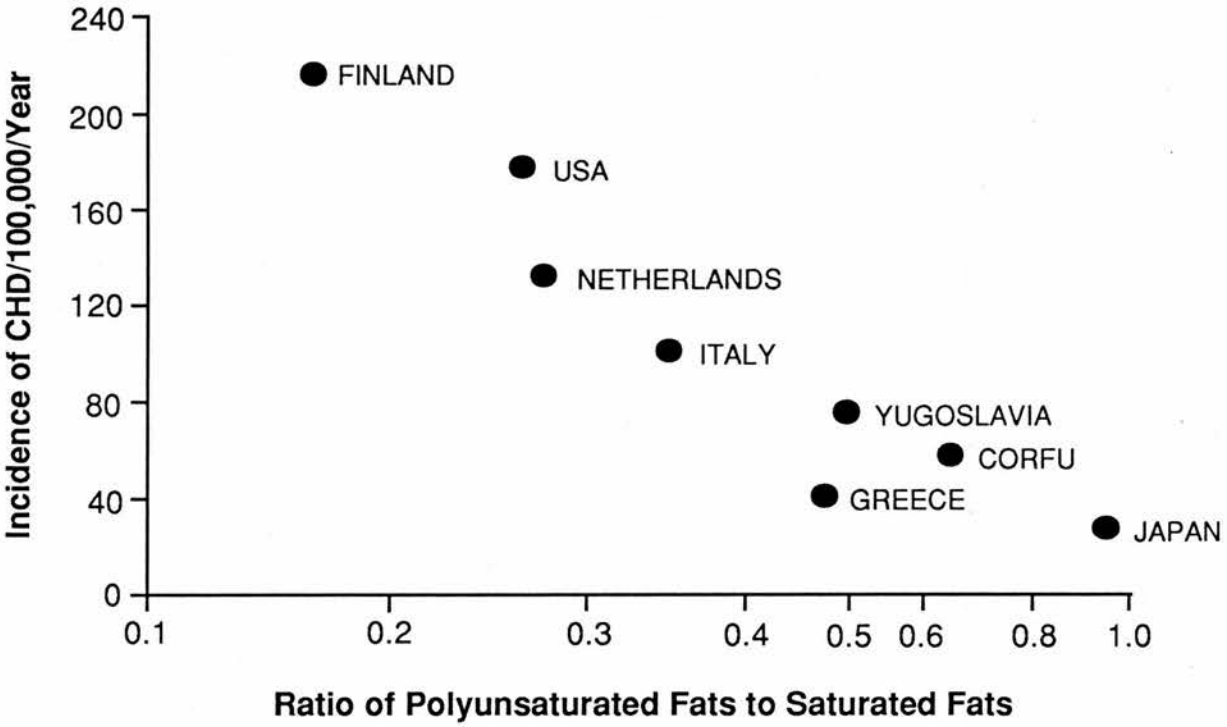
Experiments in animals have identified a possible antiarrhythmic effect of the essential fatty acid linoleic acid [39] [40] [41]. After feeding animals standard laboratory diets supplemented with vegetable oil rich in (n-6) polyunsaturated fatty acids the incidence of ventricular fibrillation due to acute coronary artery ligation was found to be reduced.

Dietary recommendations by the World Health Organisation [42] and the COMA Committee [43] stated that a reduction in total and saturated fat intake was desirable. There were no recommendations regarding

polyunsaturated fatty acids. The Seven Countries Study [5] identified a relationship between low P/S ratio and coronary heart disease mortality (Figure 1.3). The effect was assumed to be solely due to reduced saturated fat, which caused a reduction in serum total cholesterol and inturn a reduction in coronary heart disease. However, the link between cholesterol and coronary heart disease remains controversial [44] [45] [46] and the decline in heart disease observed in the USA may be in part due to an increased P/S ratio, and increased polyunsaturated fat intake, not decreased total and saturated fat.

Further work confirming linoleic acid was definitely responsible for the reduction in VF in the animal experiments using isocalorific diets could provide an ideal low cost method for reducing mortality from sudden cardiac death in man.

FIGURE 1.3 Ratio of polyunsaturated to saturated fats and the incidence of coronary heart disease (per 100 000 population per year) in men aged 40-59 in the Seven Countries Study [5] (Greece = Corfu and Crete, but as data for polyunsaturated fatty acids for Crete were not reliable, data for Corfu byitself are also presented)

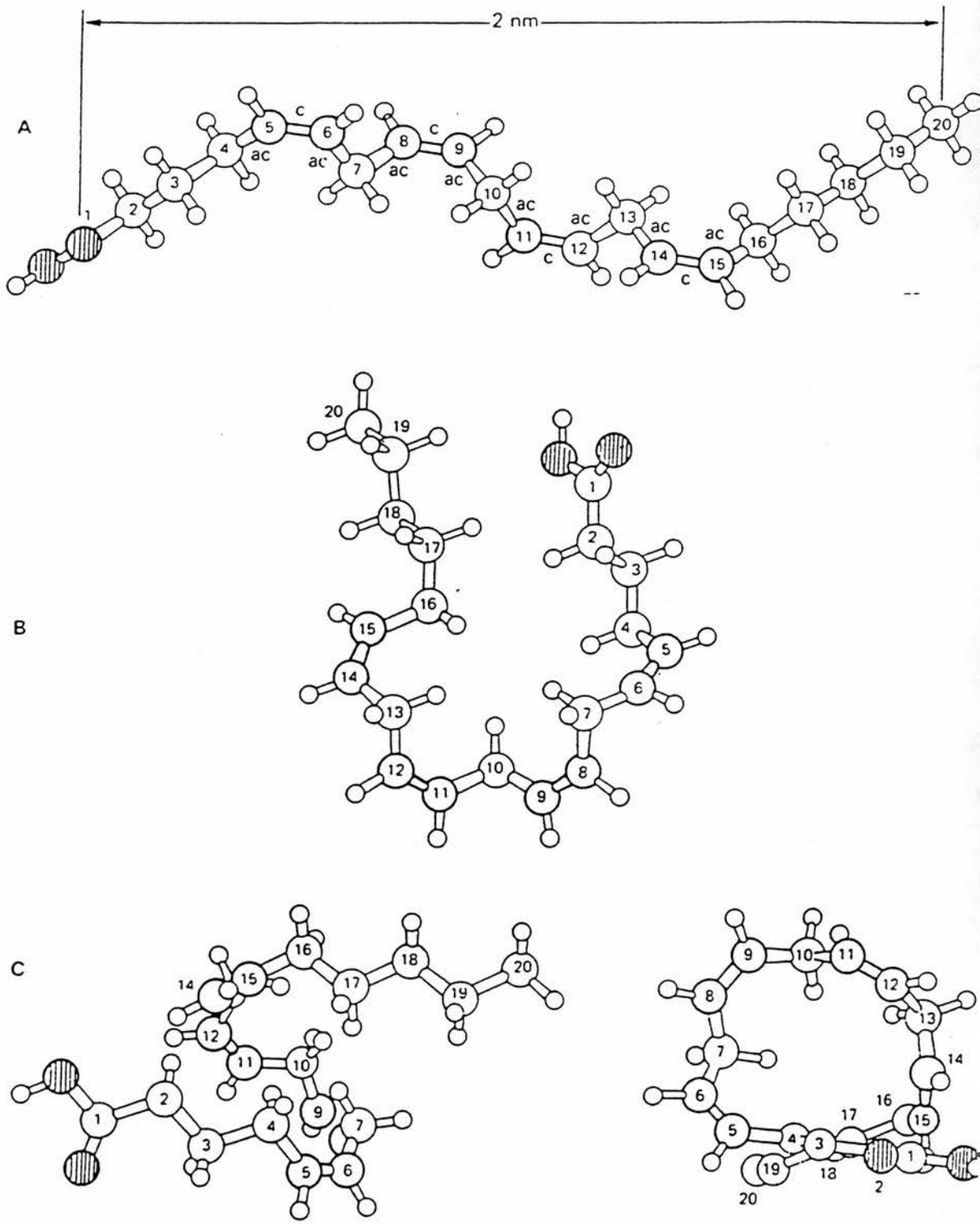


1.3 Dietary fat.

In recent years dietary fathas been linked to various diseases including cancer and heart disease [47] [48] [49]. This has led to a great deal of conflicting advice on what one should or should not eat. Fat is required by the body as an **energy source,** and as a precursor for the **biosynthesis of membranes** and **prostanoids.**

Dietary fat is consumed predominantly as triglyceride. Every triglyceride consists of three glycerol hydroxy groups each esterified with a fatty acid. The fatty acids are a combination of saturated, monounsaturated or polyunsaturated molecules (throughout the thesis the "(n-*)" nomenclature is used for fatty acids [50]. This method specifies the number of carbon atoms and double bonds and identifies the position of the first double bond from the methyl terminus of the fatty acid). All the double bonds are cis and are separated one from another by a single CH_2 group. These two features evoke a large variety of possible molecular conformation increasing with the number of double bonds present. The number of possible conformations which may be assumed by arachidonic acid have been calculated by Jong et al [51] and are illustrated in Figure 1.4.

FIGURE 1.4 Some possible conformations of arachidonic acid from Jong et al [51].



Saturated, monounsaturated and polyunsaturated fatty acids are synthesised by the body (endogenously) as well as being consumed in the diet (exogenously). Mammals are unable to synthesise 18:2(n-6) and 18:3(n-3) fatty acids as they do not possess the necessary enzymes. Therefore, certain polyunsaturated fatty acids (PUFA's) can only be obtained from the diet and are essential for normal growth [52]. These are classified as the n-6 and n-3 families, which are not interconvertible (Figure 1.5). The major effects of n-6 essential fatty acid deficiency are shown in Table 1.1.

FIGURE 1.5 Essential polyunsaturated fatty acids of the n-6 and n-3 families.

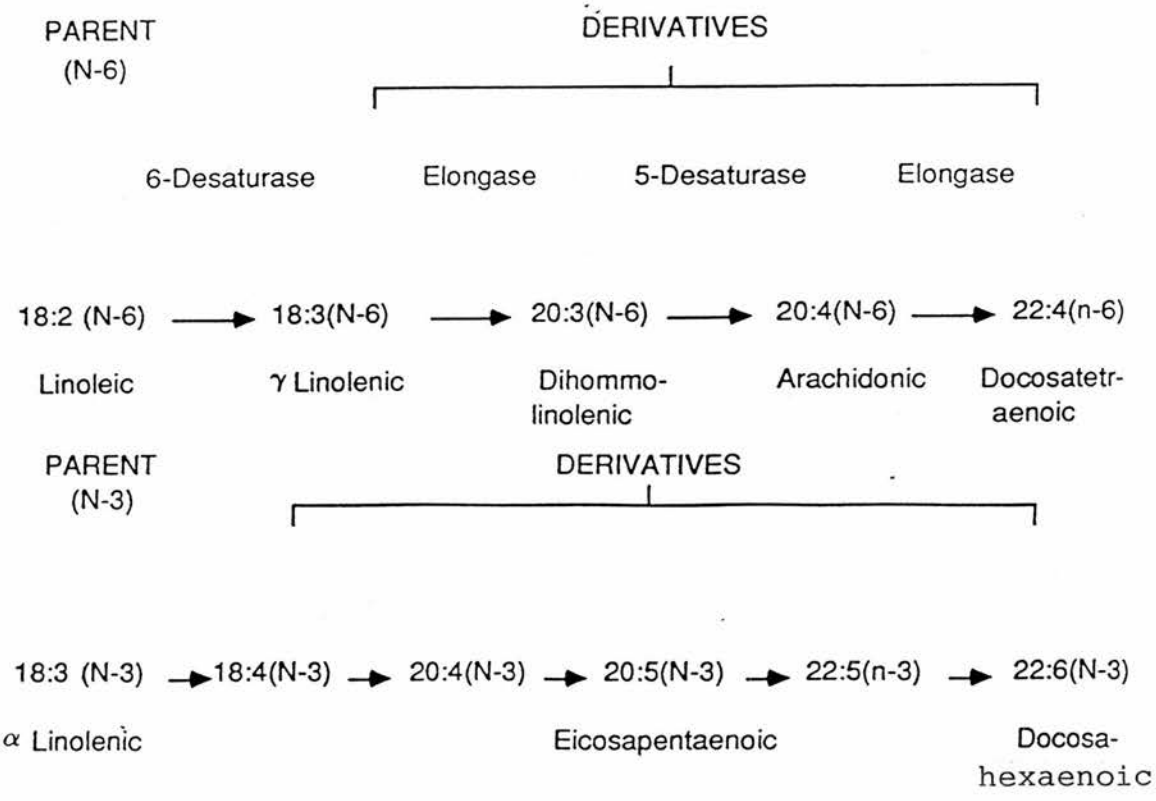


TABLE 1.1 The major effects of n-6 EFA deficiency in the rat.

1. Skin symptons	Dermatosis; increased water permeability; drop in sebum secretion; epithelial hyperplasia.
2. Weight	Decrease.
3. Circulation	Heart enlargement; decreased capillary resistance; and increased permeability.
4. Kidney	Enlargement; intertubular haemorrhage.
5. Lung	Cholesterol accumulation.
6. Endocrine glands	(a)Adrenals. Weight decreased in females and increased in males.
	(b)Thyroid. Reduced weight.
7. Reproduction	(a)Females. Irregular oestrus and impaired reproduction and lactation.
	(b)Males. Degeneration of seminiferous tubules.
8. Metabolism	(a)Changes in the fatty acid composition of most organs.
	(b)Increase in cholesterol levels in liver, adrenal and skin.
	(c)Decrease in plasma cholesterol.
	(d)Changes in the swelling of heart and liver mitochondria and uncoupling of oxidative phosphorylation.
	(e)Increased triacylglycerol synthesis and release by the liver.

These effects are not alleviated by n-3 PUFA's. N-3 PUFA's were only identified as necessary for normal mammalian learning and behaviour relatively recently [53].

What controls the destination of specific fatty acids and their functions is therefore of considerable importance in the understanding of their involvement in various disease states. This section summarises the literature in this expanding area.

1.3.1 Fatty acids as an energy source.

Fatty acids in the form of triglycerides constitute a major source of fuel in animals and oil bearing plants. Triglycerides supply 38 kJoules of energy per gram [54] and are therefore a more concentrated form of fuel than either protein or carbohydrate which each supply about 17 kJ/g. As a fuel fatty acids may be required for immediate oxidation or held in reserve for future use. The liver is primarily concerned with processing glycerides for immediate usage and redistribution to other tissues for oxidation or storage. This area is not discussed, but the reader is referred to the review by Josef Patsch [55] which summarises the recent literature on dietary lipid transport.

When energy supply from triglycerides exceeds energy demand, they are deposited in adipose tissue for energy storage. Adipose tissue consists of adipocytes

bound together with connective tissue and supplied with nutrients by a network of capillaries. The adipocyte can either store fat from the circulation or synthesise its own from carbohydrate. Each cell stores triglycerides in the less bulky anhydrous form and has the capacity for a large increase in size, which cannot be achieved with glycogen energy stores.

Fatty acids are the major source of energy for the myocardium [56] as opposed to the brain which preferentially requires glucose. In mammals there tends to be a distinction between the types of fatty acids fulfilling a storage role and those involved in a structural role in membranes. In general terms, polyunsaturated fatty acids are involved in a structural role whereas saturated and monounsaturated fatty acids are sources of energy. The fatty acid composition of adipose tissue in man illustrates this phenomenon with saturated and monounsaturated fatty acids composing approximately 30 and 50% respectively whereas polyunsaturates only represent 10% most of which is 18:2(n-6) [57].

Several publications have identified low adipose tissue linoleic acid level as an independant risk factor of coronary heart disease [10] [58]. The half-life of adipose tissue fatty acids is nearly one year [59] and the relative amount of linoleic acid in adipose tissue is directly related (r=0.68) to the amount consumed [60] [61]. Because of itsaccessibility, adipose tissue

biopsy is the favoured means of measuring linoleic acid in many epidemiology studies. Follow-ups of these studies show that the low adipose levels are due to decreased dietary intake of linoleic acid [62]. The relevance of these adipose tissue findings to particular functions of the myocardium is as yet unknown. However in man there is a good relationship between adipose and myocardial 18:2(n-6) levels (RA Riemersma, unpublished data).

1.3.2 Fatty acids and membranes.

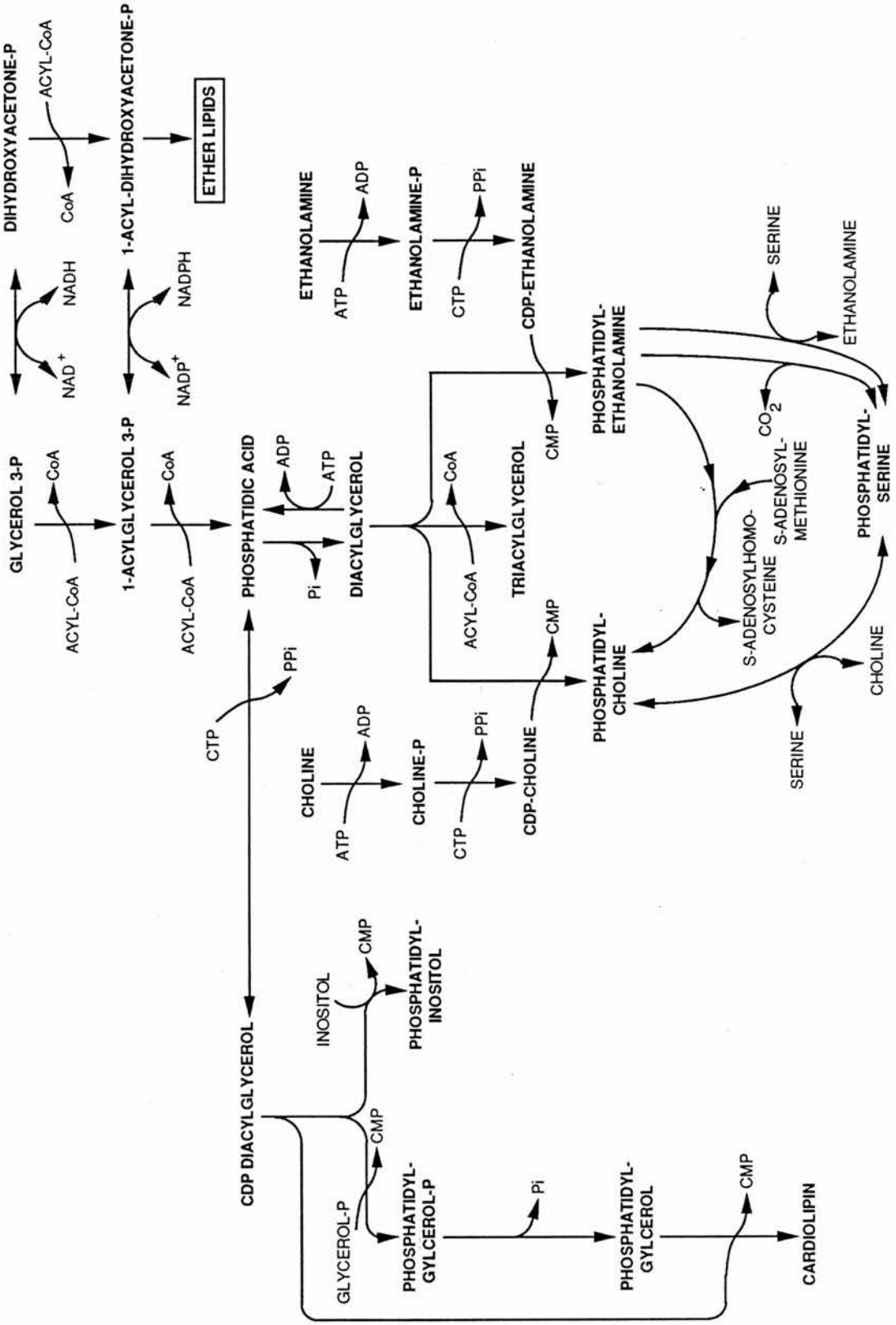
Membranes of all organelles in the body are composed of lipid and proteins. The lipid in membranes exists as phospholipids, glycolipids, and cholesterol esters. The four principal phospholipids are phosphatidylcholine, phosphatidlyserine, phosphatidylethanolamine and phosphatidylinositol. In the myocardium the phospholipid cardiolipin is also abundant. Each phospholipid molecule has a glycerol backbone with an esterified fatty acid at carbons 1 and 2, but unlike triglyceride the 3 carbon has a phosphate group with either choline, serine, ethanolamine or inositol attached to it. The fatty acids vary in chain length and saturation depending on diet and hormonal status. Unsaturated fatty acids are usually found at carbon 2, whereas saturated fatty acids are found at carbon 1. The preferential choice of polyunsaturated fatty acids

to the Sn2-hydroxyl group may be to allow the first CH_2 segment to be parallel to the surface of the membrane before assuming a perpendicular orientation within the membrane [63]. This suggests the need for the first CH_2 segment of the hydrocarbon backbone to be accessible to the outer or inner side of the membrane, possibly for cleavage by phospholipase.

The polarity of the phosphate moiety determines the transverse asymmetry of the phospholipid membrane with the hydrophobic portion of the molecule on the inside and the hydrophilic portion on the outside. In human erythrocytes 75% phosphatidylcholine, 80% sphingomyelin and 20% phosphadidylethanolamine are localised in the outer leaflet of the membrane and amino phosphoglycerides such as phosphatiylserine and phosphatidylethanolamine are preferentially present on the internal side of the membrane [64]. The asymmetric distribution of lipids in membranes has posed important questions about the mechanism of phospholipid biosynthesis, topology of the corresponding enzymes and translocation [65].

The phospholipid biosynthetic pathways of mammalian cells are illustrated in Figure 1.6.

FIGURE 1.6 The phospholipid biosynthetic pathways of the mammalian cell [66].



The base exchange between phosphatidylserine and phosphatidyl ethanolamine or phosphatidylcholine as well as the enzymatic methylation of phosphatidyl ethanolamine to phosphatidyl choline, illustrate mechanisms whereby the polarity of the phospholipid membrane can be easily modified [67] [68]. The independant exchange of fatty acids on preformed phospholipids is a further method by which phospholipids may be tailored for a specific function.

The predominant polyunsaturated fatty acid found in human phospholipid membranes is arachidonic acid, 20:4(n-6). Every phospholipid fraction has a distinctive highly conserved fatty acid pattern. For example, in rat myocardial phosphatidylserine 22:6(n-3) is the predominant fatty acid whereas in phosphatidylinositol 20:4n-6 is the major fatty acid [69]. The control and function of these specific fatty acid patterns is poorly understood. Further, the molecular species of the phospholipid fractions appears to be organ dependent, as demonstrated in PI of muscle, brain and retina by Bell et al [70].

Dietary fat can alter the fatty acid composition of membrane phospholipids. In recent years this area has been entensively studied. Data on dietary induced changes in the fatty acid composition of membrane phospholipids in various organs are now available. In this thesis only the results from heart tissue analyses are

discussed. The use of a wide variety of dietary fats of differing doses and duration in numerous animal species makes this a very confusing area. The majority of work to date has studied the effects of the dietary essential fatty acids of the n-6 and n-3 families on total myocardial phospholipids.

Diets enriched with corn oil, sunflower oil and safflower oil all contain large quantities of the essential fatty acid linoleic acid (18:2(n-6)). After feeding such diets (up to 14% w/w linoleic acid with extreme P/S ratios as large as 7.2 [71]) for varying time periods from 1 week to 20 months the myocardial fatty acid composition was altered [72] [73] [74]. However there was no change in the amounts of fatty acids incorporated into phospholipids and the P/S ratio was maintained [75] [76]. The consistent finding in all the publications was an increase in specific polyunsaturated fatty acids in total phospholipids with saturated and monounsaturated fatty acids unaltered. The greatest variation was the increase in myocardial total phospholipid linoleic acid [74] [39]. The results for arachidonic acid levels are notably inconsistent, with some experimentalists finding no changes [77] [78], while others documented increased levels [39] [79]. Many publications state that the increase in n-6 fatty acids resulted in a reciprocal decrease in n-3 fatty acids [79]. However the data on fatty acid composition

is normally presented as a percentage of total fatty acids, and the absolute amounts are rarely quoted. Only a few have examined myocardial fatty acid composition in phospholipid fractions ; PC, PE and CL. These experiments identified PC and PE as being most susceptible to dietary manipulations [80] [81]. There are no reports on the composition of the two minor phospholipid fractions; PI and PS.

The fatty acid composition of fish oil is specifically rich in the (n-3) essential fatty acids eicosapentaenoic (20:5(n-3)) and docosahexaenoic (22:6(n-3)). After feeding diets supplemented with extreme amounts of fish oil (up to 30% kcal energy from fish oil) statistically significant alterations were seen in the fatty acid composition of heart tissue phospholipids [82] [83] [84], with an increase in (n-3) fatty acids related to the amount of fish oil in the diet. 20:5(n-3) is the fatty acid which shows the most significant increase [85]. Linked to the increase in (n-3) fatty acids is a decrease in (n-6) fatty acids, specifically in arachidonic acid [86] [87]. The mechanism causing the reduction in arachidonic acid is not that of simple substitution and is discussed in section 1.3.3. The PE phospholipid fraction has been documented to be most susceptible to dietary fat manipulation [88], but again no data is available on the effects of fish oil diets on PI or PS.

Diets enriched with saturated fatty acids have not been as extensively studied. However some studies have been carried out with diets supplemented with coconut oil [89], palm oil [90] and sheep perirenal fat [91]. The results of such experiments are inconclusive, as often there is no true control group to compare the fatty acid compositions to. However, a trend towards an increase in myocardial phospholipid saturated fatty acid composition is observed. Experiments with diets enriched in rapeseed oil, which is rich in the mono-unsaturated fatty acid erucic acid (22:1(n-9)) have also been investigated [89] [92]. Such diets have been found to increase the levels of 22:1(n-9) in the myocardium phospholipid and cause myocardial necrosis [93] [94].

The results show that a complex interaction exists between dietary (n-3) and (n-6) fatty acids. This is primarily the result of competition for the same elongation desaturation enzymes. Until a full understanding of the control process in this system is known a true understanding of the mechanisms and biological consequences of dietary fat manipulations will not be fully achieved. Nonetheless the identification of an alteration in myocardial function after dietary feeding has driven many workers to investigate the functional consequences of the changes described in myocardial phospholipid fatty acid composition after feeding diets

enriched in (n-3) and (n-6) fatty acids.

One of the major areas under investigation is the interaction between membrane phospholipids and proteins. The function of a specific protein can be modulated by the lipid milieu in which it exists. Several enzymes have been identified whose function can be altered in-vitro by different fatty acids in the phospholipid membrane [95] [96]. Studies have also shown an effect on protein function after dietary fatty acid manipulations and have [97] [98]. However a full understanding of the implications of dietary fat manipulations is still far from clear.

1.3.3 Fatty acids and prostanoids.

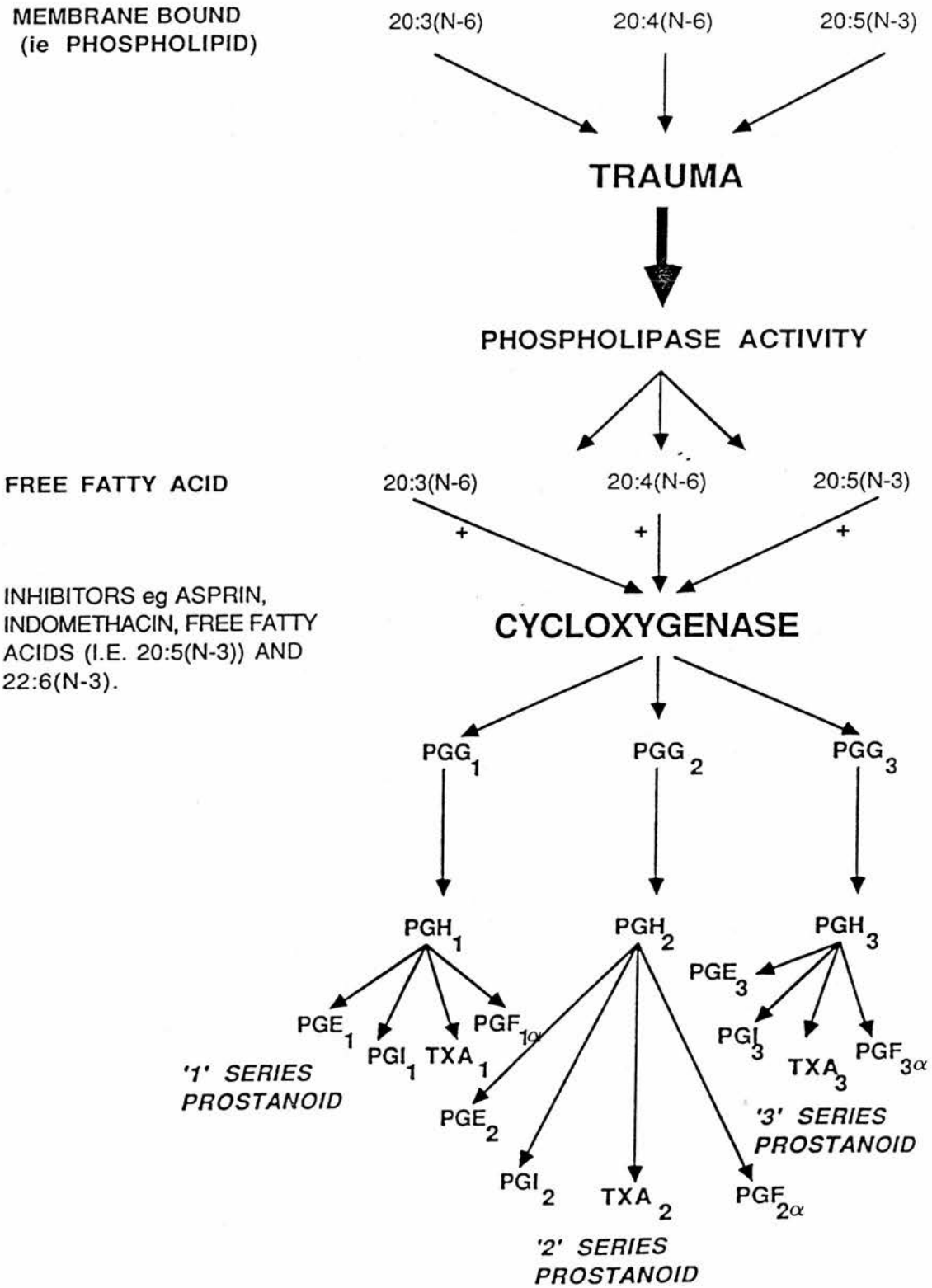
Prostanoids are a group of compounds with remarkable pharmacological and physiological properties derived from C_{20}- triene, -tetraene and -pentaene fatty acids of which those based on arachidonic acid are the most entensively studied. The specific fatty acid precursor (20:3(n-6), 20:4(n-6) or 20:5(n-3)) determines the particular series of prostanoid to be synthesised, the 1-, 2- or 3- series. These precursors are obtained directly from the diet or by elongation/desaturation of essential polyunsaturated fatty acids present in membrane phospholipids (Figure 1.5).

Prostanoids are not stored in mammalian tissue and their release from organs is therefore indicative of de novo synthesis. The type and proportion synthesised is

dependent on the situation, the tissue and the conditions at the time. Each series is synthesised via a single pathway (Figure 1.7) which produces a variety of prostanoids. The compounds have half lives of minutes or less and are then metabolised rapidly to less active compounds [99]. This particular property has made the study of these compounds difficult, with measurements relying on the assay of stable metabolites. Recent developments have improved analysis using in particular the chemical stabilisation of the prostanoids on collection [100].

Arachidonic acid is the most common prostanoid precursor in the myocardium, with minimal synthesis of the '1' and '3' series. The major metabolite released from the isolated perfused rat heart (endothelial cells plus myocytes) is prostacyclin, whereas platelets are the major site of thromboxane production. In vitro experiments have given conflicting results on prostacyclin production from isolated myocytes [101]. The discrepancies appear to be related to possible contamination of the cell preparations with endothelial cells, as recent experiments with improved techniques have identified no production of prostacyclin from myocytes in culture [102].

FIGURE 1.7 Prostanoid biosynthetic pathway.



Prostacyclin and thromboxane have opposing effects on platelet aggregation, prostacyclin inhibits, whereas thromboxane promotes action [103]. Involvement of this function has been proposed in the development of thrombi and atherosclerotic plaques. The relative balance between the two has been implicated in the development of coronary heart disease and is the basis from which the clinical thrombosis trials involving aspirin developed.

Increased bleeding time in fish eating populations [104] was proposed to be the result of a switch in prostanoid production from the '2' to '3' series. However, although there is some substitution the increased time for platelet aggregation appears to predominantly via the inhibition of the synthesis of the '2' series by increased 22:6(n-3) in the diet [105]. Increases in prostanoid production have been found between borderline EFA deficient diets and linoleic acid rich diets [73]. No dose response effect has been observed with increasing dietary linoleic acid, and if a 'mega' dose is consumed prostanoid production is in fact inhibited [48].

Prostanoid involvement in arrhythmias has been studied and has produced a large amount of conflicting results [106]. The reasons for such discrepancies are probably differences in species, anaesthetic, vehicles used for administration, doses and routes of

administration. Furthermore, the short half-life of the active compound makes pharmacological experimentation difficult. Prostacyclin or its stable analogue iloprost and PGE_2 are antiarrhythmic in animal experiments using occlusion of the left anterior descending coronary artery as the experimental model [106] [107]. Prostanoid inhibitors have also been used for investigations, but again the results are contradictory, with an effect appearing to intimately depend on the dose of drug used. In general, indomethacin, iboprofen and flurbiprofen have all been reported to exacerbate arrhythmias at high doses, while reducing them at low doses. In contrast aspirin appears to have an antiarrhythmic effect at both high and low doses.

Prostanoids have been proposed to be the mechanism whereby (n-6) and (n-3) fatty acids exert their essentiality for normal cellular function. However, prostanoid are formed in exceedingly small amounts in vivo and would require to be turned over metabolically at an impossibly high rate to account for the large dietary requirement for (n-6) PUFA in mammals (in the region of 2-4% of the total calorific intake per day in man). In conclusion, the highly active prostanoids appear to be intimately involved with the development of arrhythmias, but the full implications of these actions have still to be realised.

1.4 Arrhythmias.

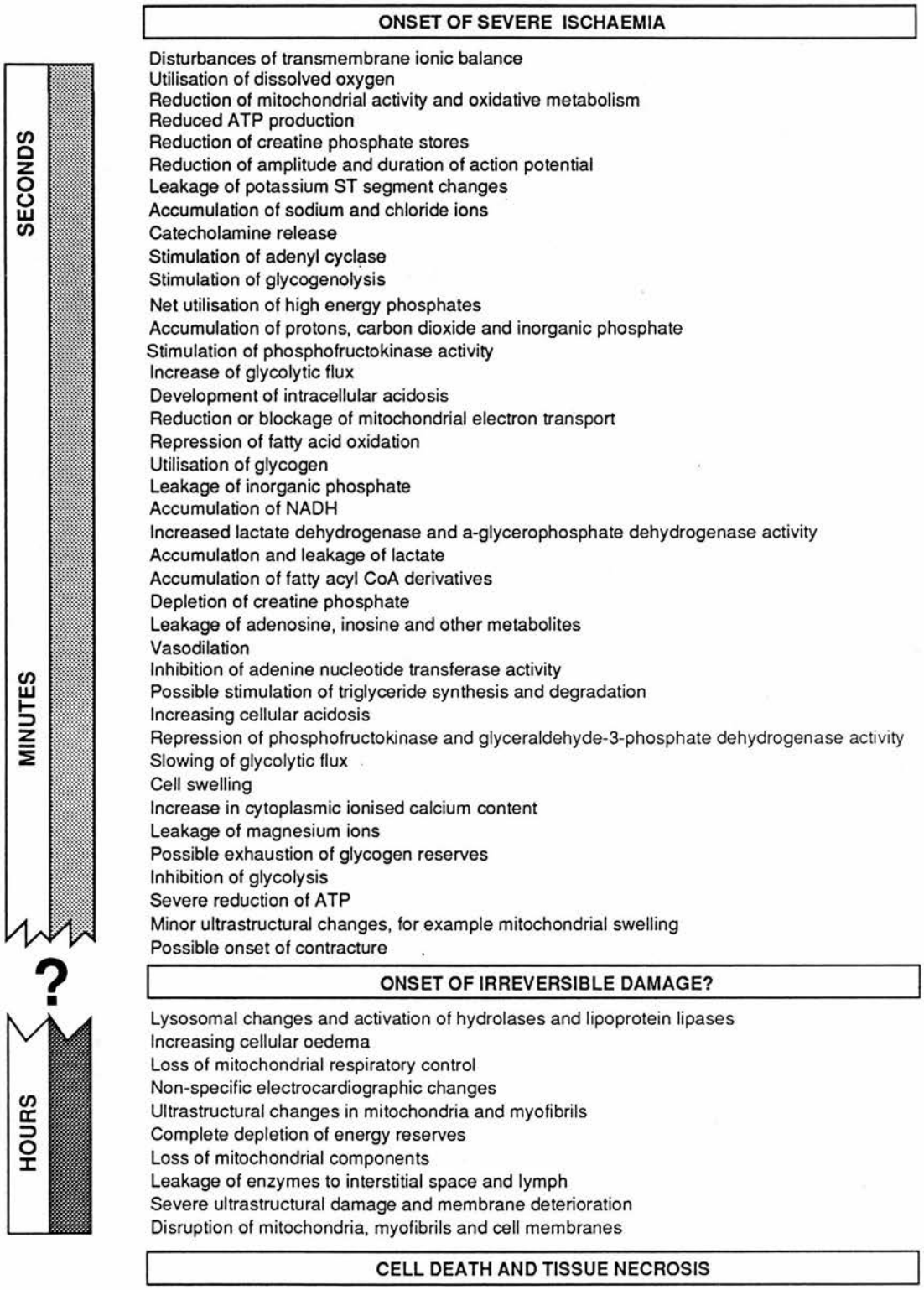
1.4.1 Experimental models for the study of arrhythmias.

There are two approaches to the study of arrhythmias. One is to mimic the situation observed in man and the other is to use substances which have been found to induce arrhythmias.

The occurrence of an ischaemic episode is the major cause of arrhythmias [108]. A large number of different experimental models have been developed to study the consequences of myocardial ischaemia. Before discussing the differences among the various experimental models, the biochemical and physiological consequences of ischaemia are briefly summarized.

Ischaemia is defined as a lack of blood supply to a particular tissue. This results in a deficit of oxygen, substrate and energy supply as well as reduced removal of potentially toxic waste metabolites such as lactate, carbondioxide and protons. The events responsible for the ischaemic event were briefly discussed in section 1.1. The onset of ischaemia results in a number of deleterious changes which are summarized in Figure 1.8. The events depicted are not intended to occur in sequential order, but should be thought of as a dynamic process.

FIGURE 1.8 Events occuring after the onset of myocardial ischaemia.

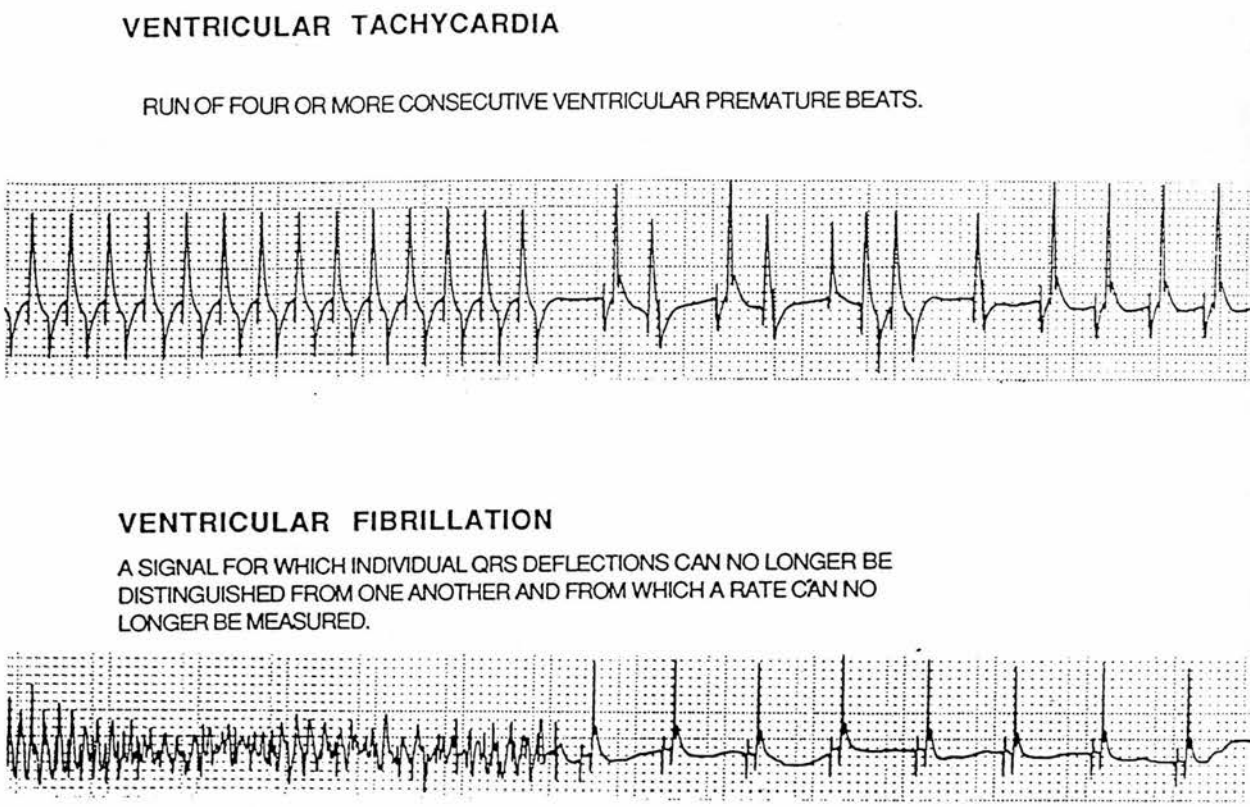


Two types of ischaemia can be induced experimentally, global or regional. Clinically global ischaemia (i.e. of the whole heart) is rare, but is observed during cardiac surgical procedures, such as cardiopulmonary bypass where the aorta is cross-clamped. Regional ischaemia is assumed to mimic the clinical situation in patients with coronary heart disease. In animals regional ischaemia is induced by temporary or permanent occlusion of one or more healthy coronary arteries (spontaneous atherosclerosis is rare in most experimetal animal species). Two methods can be used to occlude, stitching and tying coronary arteries or insertion of an intracoronary electrode. The latter method uses electrical stimulation to induce thrombus formation and hence ischaemia. This method reproduces the potentially gradual or intermittent development of intracoronary thrombus found clinically in man. However, limited control over the severity and onset of ischaemia make it less useful to monitor the suitability of various therapeutic interventions, and is for obvious reasons not technically possible in small animals. Therefore, although not an exact replicate of the clinical situation stitching of the coronary artery is the method most frequently chosen by experimentalists to assess the viability of an anti-arrhythmic effect.

The choice of in-vivo or in-vitro conditions

depends on the purpose of one's proposed studies. In the context of this thesis, the in-vitro Langendorff was preferred. Earlier studies in-vivo suggested an antiarrhythmic effect of diets enriched with linoleic acid. Therefore the principal reason for the choice was to confirm previous antiarrhythmic findings in-vitro **without** the influence of circulating factors such as platelets and removing some of the influences of the intact nervous system. This method had the further advantage of being reproducible and technically easier to perform allowing the study of large numbers of animals, thus increasing the statistical power of the studies. Information on coronary flow and myocardial metabolism are not obtainable from in vivo experiments but are measurable in the in-vitro system. The effect of drugs is directly on the heart and would not be influenced by an effect on another organs or circulating factors in the intact animal. The one major disadvantage of the method is that in the absence of blood pressure measurement, (available in the in vivo model) which aids identification of VF and VT which can be difficult. This problem can be overcome by enforcing the guidelines of the Lambeth Convention [109]. The convention states that "randomisation to treatment and blind analysis are essential" to prevent biased analysis. At the start of all experiments, the classification of VT and VF must be clearly defined (Figure 1.9).

FIGURE 1.9 Examples of the classification of arrhythmias.



The incidence of arrhythmias can be low in animal models, even after ischaemia is induced. Therefore, many experimentalists modify conditions to increase the incidence on occlusion. Two methods are most commonly used, the alteration of potassium levels [110], [111], or the use of animals which are older [112]. The incidence of ischaemic arrhythmias is well documented to increase with age [113] [112]. Throughout this thesis potassium levels in the perfusate were reduced from the physiological concentration in the rat of 6.1

mM to 3.0 mM [114]. Other experimentalists have employed the use of diuretics which reduce plasma and myocardial potassium levels or feeding diets low in potassium, to animals prior to study [114] [115].

The use of compounds to induce arrhythmias is a technique whose relevance to the clinical situation is questionable. The compounds most frequently used include ouabain [116], isoprenaline [117], $CaCl_2$ [118] and arachidonic acid [119]. The compounds are injected intravenously and the mechanism of 'sudden death' is frequently unknown. Therefore, several factors may be involved and the cause of death might be extracardiac. Indeed, arachidonic acid was assumed to cause sudden cardiac death, but in one study employing histopathology lung embolism was subsequently found to be the true cause of death [119]. Therefore, although these methods are very simple, evidence of their mode of action must be an absolute prerequisite.

1.4.2 Essential fatty acids and arrhythmias.

The first in vivo antiarrhythmic effect of linoleic acid rich diets was reported by Szekeres et al in 1980 [39]. The feeding of standard chow supplemented with 12% w/w sunflower oil to 12 week old male rats resulted in a significant reduction in both VF and VT when compared to the standard laboratory chow (5% fat). The results did not discount any influence from reduced mineral or vitamin intake as a result of an imbalance

in calorific intake. The diets used were extreme, a P/S ratio of 6.5 for the sunflower diet, which could not be achieved by dietary modification of the average British diet. Therefore the clinical relevance of these results is questionable.

Other research groups have published the effects of linoleic acid rich diets on the incidence of ischaemic VF both prior and after Szekeres's publication. An earlier experiment by Logan et al [118] using $CaCl_2$ to induce arrhythmias compared two semi-synthetic diets with 20% w/w safflower oil or beef dripping and demonstrated no antiarrhythmic effect. The incidence of VF after a feeding period of one month was 45% in the safflower oil group and 50% in the beef dripping group (not significant). Linoleic acid levels in myocardial triglycerides increased (5% to 44%), however the results are only on the basis of one analysis. The numbers reported were small (n=10 per group) and insufficent to base any solid statistical analysis with a control incidence of 50%.

An Australian group headed by Dr J Charnock has published a great deal of work in this area in recent years. In 1985 they published an antiarrhythmic effect of linoleic acid rich diets [40]. The experimental diets used were fat supplemented chow with both the fat content and composition more realistic than Szekeres (16% w/w fat and P/S ratios of 0.2 and 3.6). Standard

laboratory chow (4% fat) was the control. The difference in calorific intake resulted in an average consumption of the diet in the oil supplemented groups of 20gm/day as opposed to 23gm/day in the reference group. Therefore the dietary mineral intake by the rats differed. The vitamin E content of the diets were uncontrolled, therefore an influence from this potentially anti-arrhythmogenic factor [120] could not be removed. The numbers of animals per group were very small (n=5) therefore statistical conclusions should not have been drawn. In this study in contrast to other publications the length of time the animals were fed diet was much longer, 7 and 20 months as opposed to 1 and 3 month feeding periods used by Logan and Szekeres. More recent publications from this Australian group have illustrated an antiarrhythmic effect during both ischaemia and reperfusion with fish oil supplemented diets [121]. However the same critisms on the use of unbalanced diets and group sizes remain even with the number of animals per group increased to ten.

One other group has recently published on the effect of linoleic acid rich diets in the isolated perfused working heart [72]. The diets used by this group were purified and semi-liquid and extremely unrealistic if extrapolated to man (P/S ratio of 5.3 (sunflower seed oil) and 0.001 (coconut oil). Furthermore the diet rich in saturated fat (hydrogenated

coconut oil) could have been EFA deficient. The number of animals screened for arrhythmias was again small (n=12). After 1 week of feeding these diets there was a change in myocardial phospholipid fatty acid compositions between the two groups. The perfusion results were expressed as an overall arrhythmia score (i.e. VPB's, VT and VF). There appeared to be a trend towards a reduction in the incidence of VF in the linoleic acid rich diet but, no statistical analysis had been carried out, presumably because of the small numbers.

The work described above indicates that linoleic acid rich diets could be antiarrhythmic. However, there are various shortcomings in all of the experiments. Without exception, the experiments used too few animals. None of the experimental diets correctly controlled the vitamin and mineral contents. The absence of an antiarrhythmic effect in Logans experiments with equally small numbers could indicate that linoleic acid is not antiarrhythmic if controlled semi-synthetic diets are used.

As was mentioned the effect of fish oil diets on ischaemic VF have also been studied by two other groups and have given inconclusive results. The first study was carried out in 1980 by Culp et al [122] in dogs. The experimental diet used contained large amounts of fish oil (25% Kcal) and was given over a period of 36-

45 days to a group sizes of only 10. The control group was given standard uncontrolled dog chow (no fat supplement, therefore not isocalorific) for 1-7 days. Intracoronary electrodes were used to induce acute coronary thrombosis and subsequent arrhythmias, therefore all the problems discussed in the previous section could be levelled at this study, specifically as fish oil is believed to have anti-thrombotic properties [123]. The incidence of VF was not significantly different between the two groups (control=29% and fish oil=30%). However, a reduction in the mean frequency of ectopic beats from 80% in the control group to 30% in the fish oil group was reported. The reduction was assumed to be due to a reduction in infarct size (3 $\pm$ 1 vs 25 $\pm$ 4%). However, the proportion of the left ventricle deprived of blood was smaller in the fish oil group (no data presented). This difference in myocardium at risk therefore acts as a confounder in the experiment, and the control group with the greater occluded zone would automatically have been expected to be more succeptible to VF [124].

The other report was from Holland by Hartog et al in 1986 [125]. This experiment investigated the effect of fish oil diets on ischaemic arrhythmias in piglets. The diets employed were identical in every factor bar their fatty acid composition and subsequent P/S ratio's. Like all the others large amounts of fish oil

(23.9 kcal), which could never be consumed in man were used. The results presented for VF were the result of six 5-minute periods of ischaemia, induced by clamping the left anterior descending coronary artery, followed by a 10 minute period of reperfusion. No significant differences were seen between the two groups. However, it should be noted that the group sizes were very small (n=8) and that reperfusion and ischaemic VF were not distinguished (even though the mechanism of the two are probably different).

In conclusion, the effect of linoleic acid and fish oil on the incidence of ischaemic arrhythmias has not been studied effectively. Isocalorific semi-synthetic diets and the use of the Langendorff model with carefully controlled K^+ would allow the study of larger numbers of animals and remove the short comings of previous experiments. In addition the possible mechanisms responsible for the anti arrhythmic effect could be narrowed down by removing the influence of circulating factors (platelets,lipids, etc) present in the in vivo model.

HYPOTHESIS

Diets enriched in essential fatty acids reduce the incidence of ventricular fibrillation during acute myocardial ischaemia by altering the fatty acid composition of myocardial membranes.

CHAPTER 2

METHODS

2.1. - Animal housing conditions.

Male rats were used throughout and the choice of strain is discussed in Chapter 3. Four animals were kept per cage and all animals for an experiment were housed in one room. The ambient temperature was 20-21^{o}C and the light/dark cycle was a constant 12 hours on and 12 hours off. Water and food were given ad libitum. The animals food and cages were changed three times a week and body weights taken once a week.

2.2. - Diets.

2.2.1 Diet calculation.

All diets used were semi-synthetic. Every diet was formulated from maize starch, casein, cellulose, salt mixture, vitamin mixture and experimental fat (the sources of which are presented in Table 2.1). The amounts of fat, protein, carbohydrate, salts and vitamins were calculated from Unilever Research food tables [126].

The basic diet used in all experiments had 40% energy from fat, 23% energy from protein and 37% energy from carbohydrate. Table 2.1 shows the basic ingredients for a 40% energy fat diet. When different energy percentages of fat were required the contribution of carbohydrate was modified proportionately.

TABLE 2.1 - Ingredients of a semi-synthetic rat diet (40% energy fat).

Ingredient	Supplier	% by weight
Cornflour	Fleming Howden	45.6
Casein	Milk Marketing Board/ Sigma	26.7
Cellulose	Sigma	6.5
Vitamin mix*	Various (see appendix 1)	0.43
Salt mixture	Various (see appendix 2)	2.33
Fat mixture	Various (see appendix 3)	18.5

* N.B. minus vitamin E

In the initial studies Sigma vitamin free casein (cat No.C3400) was used, but the high cost (£50 per 5kg) necessitated a cheaper source to be identified. The effect of the cheaper milk marketing board casein (Scottish Pride) on the incidence of VF was examined using the methods stated in section 2.4. The source of casein did not affect the incidence of VF (Table 2.2).

TABLE 2.2. - The effect of M.M.B. casein on the incidence of VF during acute myocardial ischaemia in isolated perfused Lew rat hearts

Supplier	% VF	%VT
Sigma (n=36)	53	97
M.M.B. (n=32)	56	97

(N.S. after Chi-square test)

The required fatty acid composition of the experimental fat was obtained by mixing edible oils. The precise mixture was calculated using a computer program solving simultaneous equations (LPGO ; Mr R H Fawcett, Agricultural Resource Management, Edinburgh University). The amount of saturated, monounsaturated and polyunsaturated fatty acids of various oils and fats were obtained by GLC analysis and/or published food tables [127]. The program calculated the optimal, most economic fat mixture from the data of the available oils. The fatty acid composition of the optimal solution was checked gas chromatographically using the methods stated in section 2.3.6 and 2.3.7.

Vitamin E deficiency has been linked to arrhythmias [120]. Oils rich in polyunsaturated fatty acids are also rich in vitamin E. Therefore, to prevent any confounding influence a constant vitamin E level was calculated for each experiment. The amounts were either adjusted to the highest level in a particular experiment or to the minimum requirement of 80 IU/kg diet [128] by the addition of d-α-tocopherol acetate (see each experiment for specific values).

2.2.2 Diet Preparation.

All diets were prepared in a specially designed diet kitchen.

Three types of balance were used throughout to weigh differing quantities of ingredients:-

(1) for weights less than 1g the Oertling balance was used.

(2) for weights between 1g and 100g the Sauter RL200 was used.

(3) for weights greater than 100g the Sauter Shandon Tara was used.

The salt mix was made using the ingredients listed in Appendix 2. The salts were mixed 1-2 minutes in the Robot Coupe and then aliquoted in 46g batches (sufficient for 2kg of complete diet) into sealed plastic containers and stored in the diet kitchen. The vitamins listed in Appendix 1 were weighed and mixed for less than 1 minute in the Robot Coupe, aliquoted into 8g batches (sufficient for 2kg of complete diet) and stored in sealed plastic bottles in the cold room.

All the dry ingredients (i.e. cornflour, cellulose and casein) were preweighed and stored together in batches required for 2kg of diet in plastic bins in the diet kitchen.

The fat mixes were prepared freshly each day in 2kg batches. The solid fats were melted, then tocopherol acetate (vitamin E) and the other oils were

added then mixed thoroughly. The fat handling and subsequent mixing was initally a slow process, involving melting the solid fats in a water bath at 50°C. In March 1988 a microwave oven was purchased. This melted the fat much more efficiently at a power setting of 9 for 3 minutes, with the temperature not increasing above 50°C. All fats were stored in the 4°C cold room.

All pre-prepared ingredients were mixed using a Robot Coupe. A maximum of 2kg was mixed at any one time. The dry ingredients plus salt and vitamin mix were mixed together for less than 1 minute in the Robot Coupe mixer. The mixer was switched on at low speed and the weighed, molten fat mix was slowly added. The complete mixture was mixed for 2 minutes at low speed and then 1 minute a high speed. The mixer was then emptied and the diet stored in the cold room. The process was repeated until sufficient diet had been made. The mixer was thoroughly washed after every different diet.

To minimise peroxidation, diet was freshly prepared every week and stored in containers under argon in a cold room (4°C). To further limit peroxidation of fresh diet was fed and cages changed every second day .

2.3 - Biochemical Measurements.

2.3.1 Lactate Measurement.

Lactate was measured in perfusion fluid less than 60 minutes after collection using a fully enzymic kit produced by Boeringer Mannheim (kit No. 256773).

The principle was a coupled assay and is shown by the following reactions:-

$$\text{L-Lactate} + NAD^+ \underset{\text{LDH}}{\rightleftharpoons} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{L-glutamate} \xrightarrow[\text{GPT}]{} \text{L-alanine} + \gamma\text{-oxoglutarate}$$

Lactate was measured spectrophotometrically (350nm ; 37^{o}C) as NADH produced in the conversion of lactate to pyruvate in the presence of excess NAD^+. The equilibrium of the second reaction lies far to the right and drives the complete conversion of the first reaction.

The reagents used were as stated in the kit except a 60% lower NAD^+ concentration was used, as the kit was designed for clinical samples with a much greater concentration of lactate. The standard used was a triplicate measurement of 1mM L-lactate.

A Cobas Bio centrifugal analyser (Roche Diagnostics) was used for all the measurements. To allow comparisons both between and within studies Precinorm S (Boeringer) was used as a quality conrol (nominal mean

concentration 880uM/L). The inter assay coefficent of variation of lactate was determined by repeated analysis of Precinorm S throughout all the studies (Table 2.3). Two values are given for inter assay because the batch of quality control was changed one year into the study. Lactate is expressed as uM produced / minute / gram wet weight of heart throughout.

TABLE 2.3 - Coefficients of variation of lactate.

	Coefficient of Variation
Intra assay	0.879 %
Inter assay (within study)	6.4 %
Inter assay (between studies)	
1/10/86 - 1/11/87	3.0 %
2/11/87 - 31/9/89	10.1 %

2.3.2 Lactate dehydrogenase (LDH) measurement.

LDH activity was measured using the optimised standard method produced by Boehringer Mannheim (kit No. 543039). The principle of the measurement was the following forward reaction:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightleftharpoons[\text{LDH}]{} \text{L-Lactate} + NAD^+$$

Therefore, LDH was measured spectrophotometrically at

340nm ; 37°C by the disappearance of NADH. All solutions were as described in the kit. The activity was measured on a Cobas Bio centrifugal analyser and the results were calculated from the change in optical density. LDH has been documented to be unstable in perfusion fluid, so its stability was monitored over a period of 2 hours (Figure 2.1.). The results showed that all samples were stable up to 2 hours after collection whether stored at 4°C or room temperature. All samples were stored at 4°C and measured within 2 hours of collection throughout the study.

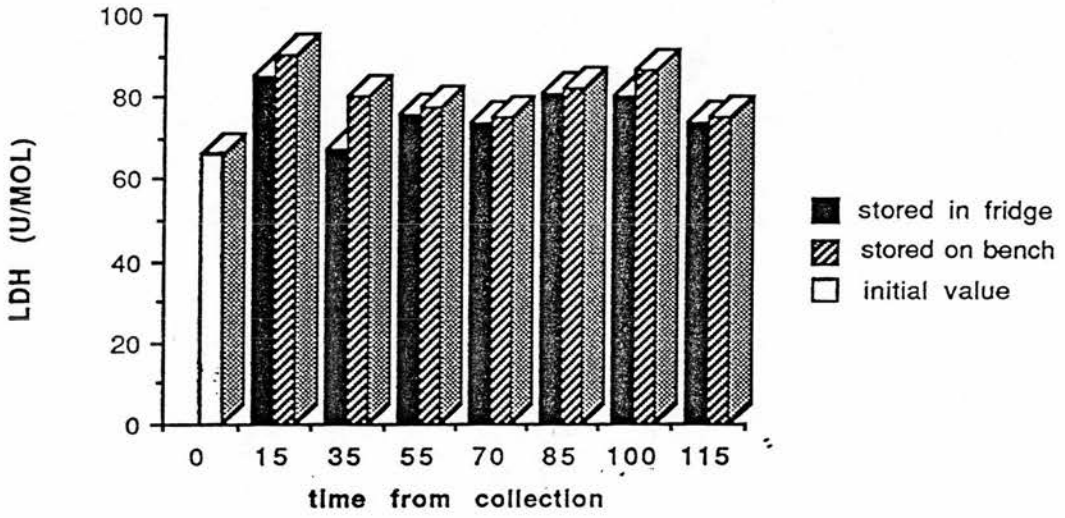
Intra and inter assay quality controls were monitored using Precipath UBS (Boeringer ; mean concentration 445 U/l). The coefficents of variation were determined by repeated analysis of Precipath UBS throughout all the studies (Table 2.8)

The results are expressed in Units/minute/gram wet weight heart throughout.

TABLE 2.8 - Coefficient of variation for LDH measurement.

	Coefficient of Variation
Intra assay	3.1 %
Inter assay (within study)	7.5 %
Inter assay (between studies)	10.4 %

FIGURE 2.1. - Stability of LDH in perfusion fluid over a 2 hour period after storage of the samples at $4^{o}C$ and room temperature.



2.3.3 Analysis of total and fractionated myocardial phospholipids.

Total and fractionated phospholipid analyses were carried out in batches of eight hearts plus a blank (the hearts were randomly selected).

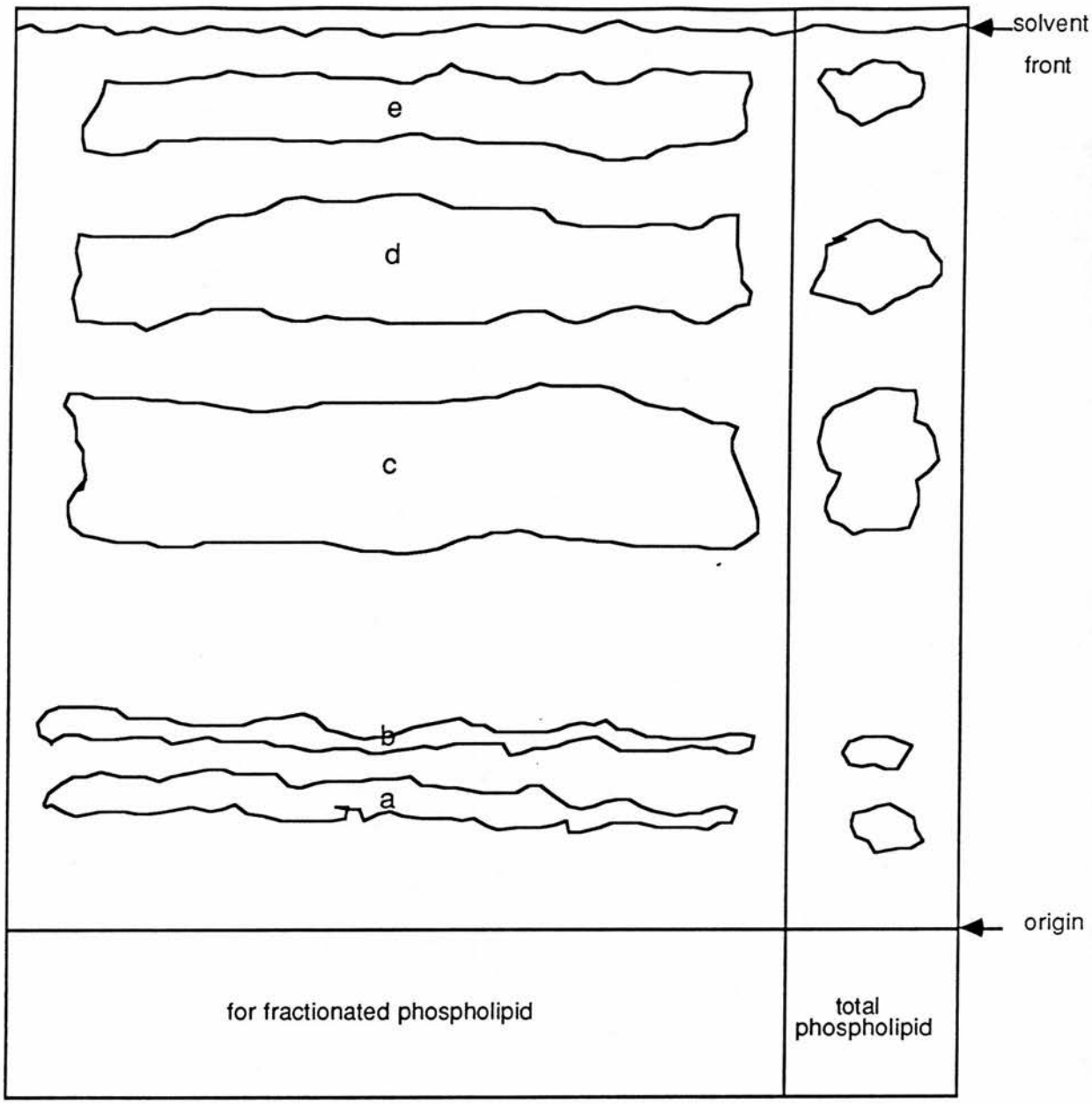
Hearts were freeze dried for 48 hours on an Edwards Modulyo freeze drier. Each heart was crushed into a fine powder using a mortar and pestle. The powder (less than or equal to 145mg) was weighed and

transferred into Quickfit tubes (20 x 125 mm).

The phospholipids were extracted according to the method of Folch [129] using 21 ml chloroform/methanol (2:1 v/v). The lipid extract was dried down under vacuum in a 1 l Buchi flask using a Buchi-R rotary evaporator and a water bath at 30°C. The lipid extract was redissolved in 2 ml methanol and 4 ml chloroform and placed in a Quickfit tube. The extract was dried down under vacuum and redissolved in 1.0 ml of chloroform/methanol (2:1 v/v) using a Hamilton syringe.

Phospholipids were separated by T.L.C. using LK5 plates with a preadsorptive area (Whatman catalogue no. 4855 820 (20 x 20 cm)). The plates were impregnated with 1.2 % boric acid and reactivated at 100° (60 min ; [130]. One plate was used per sample to separate both total and fractionated phospholipids. One hundred micro litres of extract was spotted for total phospholipid and a 100 ul was streaked across the remainder of the plate for fractionated phospholipids (Figure 2.2). The plates were developed in $CHCl_3$: MEOH : H_2O : NH_3 (120:75:6:2 v/v/v/v) in a paperlined tank at room temperature. The plates were left to run for ~ 1 hour, air dried and sprayed with 0.1% dichlorofluorescein. This allowed the phospholipids to be visualised under ultraviolet light at 365 nm. A typical separation is shown in Figure 2.2.

FIGURE 2.2 Separation of myocardial total and fractionated phospholipids.



a = phosphatidylinositol

b = phosphatidylserine

c = phosphatidylcholine

d = phosphatidylethanolamine

e = cardiolipin

individual bands scraped for each fraction

All 5 bands were scrapped for total phospholipid

The position of the bands using this particular separation system had been previously identified using known standards. The bands for total phospholipid and each of the 5 fractions were scraped off and placed into individual Quickfit tubes.

Before transmethylation internal standard was added to every tube (the standard could not be added earlier as specific standards for each phospholipid fraction are not commercially available). The concentration of the standard depended on the particular phospholipid fraction in the tube :-

a. For total phospholipid , PE , PC and CL 70ug phosphatidylcholine C17:0 was added.

b. For PI and PS 2ug Phosphatidylcholine C17:0 was added.

Base-catalysed transmethylation was carried out as described by Christie [131] using toluene and sodium methoxide (0.5M).

The methylesters were redissolved in 25ul chloroform with 0.01% BHT and stored in GLC vials at -20^{o}C prior to GLC analysis. The results are expressed as ug fatty acid /gram wet weight of ventricular heart tissue.

2.3.4 Analysis of myocardial triglyceride fatty acids.

Triglyceride fatty acid analysis was organised as for phospholipid fatty acid analysis in section 2.3.3.

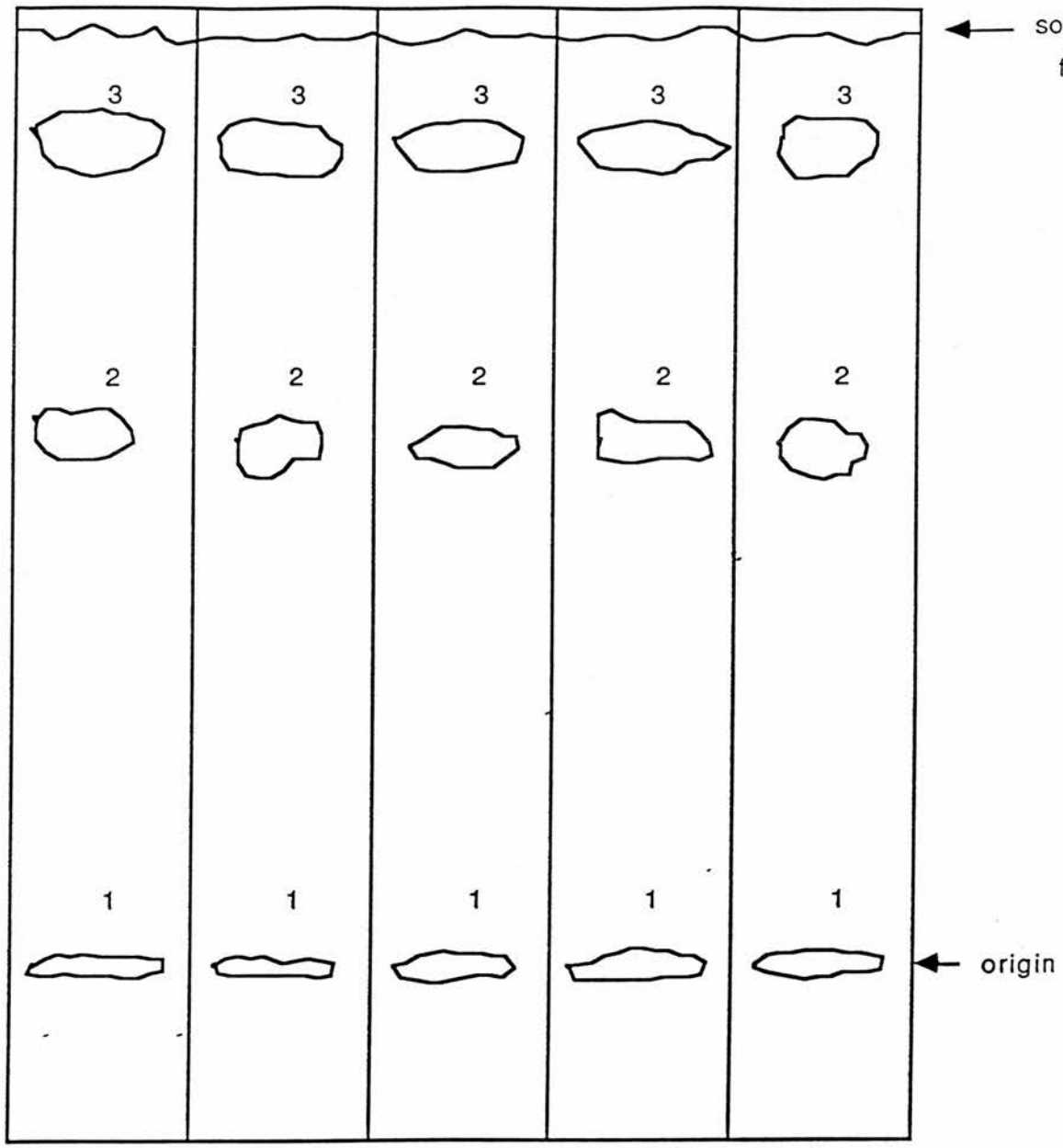
Hearts were extracted as in section 2.3.3. One

fifth of the final lipid extract (200 ul) was transferred to a Quickfit tube and 40 ug of internal standard (triheptadecanoin) was added . The lipid fraction was dried down and redissolved in 1.0 ml of chloroform:methanol (2:1 v/v).

200 ul of lipid extract was spotted on Merck 5721 plates in 5cm bands. The plates were predeveloped in hexane:diethylether:formic acid (80:20:2 v/v/v) for ~45 minutes. The plates were air dried and sprayed with POPOP/PPO to allow identification under ultraviolet light at 365 nm. Figure 2.3 shows a typical separation. The triglyceride band, previously identified using known standards and was scraped off and placed in a Quickfit tube.

Transmethylation was carried out as in section 2.3.3. The methyl esters were redissolved in 25 ul chloroform with 0.01% BHT and stored at -20^{o}C prior GLC analysis. The results are expressed as ug fatty acid per gram wet weight of ventricular heart tissue.

FIGURE 2.3 Separation of myocardial neutral lipids.



1 = total phospholipid

2 = triglyceride

3 = cholesterol esters

2.3.5 Analysis of adipose tissue fatty acids.

One experiment contained a maximum of forty samples including a blank and a control.

Approximately 10 mg of adipose tissue from the inguinal region of the rat was homogenised in all-glass Elvjehem Potters using 5 ml isopropanol/heptane (4:1 v/v). Phospholipids and free fatty acids were back extracted using 3ml KOH (0.05 %) and the triglyceride remained in the top organic phase. This was then washed with 8 ml of isopropanol/KOH (0.05% ; previously washed in heptane (4:1:3 v/v/v)). The heptane layer was transferred and evaporated to dryness using the Buchi (see section 2.3.3).

Transmethylation was carried out as in section 2.3.3. The methyl esters were redissolved in 40 ul of chloroform with 0.01 % BHT and stored as in section 2.3.3.

The results were expressed as a percentage of the total weight of the fatty acids. Calculation of the coefficient of variation was determined by repeated analysis of a single large sample of human adipose tissue. An aliquot was analysed with every study, which gave a coefficent of variation for 18:2(n-6) of 1.8%, which allowed interstudy comparisons.

2.3.6 Fatty acid analysis of oils and diets.

Lipids were extracted from approximately 80 mg of

oil or 150 mg of diet. The particular sample was weighed out and the lipid was extracted in 240 ml of chloroform:methanol (2:1 v/v). 15 ml of the resulting extract was then transferred to a Quickfit tube.

An internal standard of triheptadecanoin (1mg/ml) was added to the Quickfit tube. 4 ml KCL (0.88 %) was then added to remove contaminants such as protein and glycerol. The solution was then shaken, and spun in a IEC Centra-7R centrifuge at 400 G at 20°C for 5 minutes. The lipid phase was transferred to a Quickfit tube and evaporated to dryness using a Buchi rotary evaporator and a water bath at 30°C.

Transmethylation was carried out as in section 2.3.3. The methyl esters were redissolved in 200ul chloroform with 0.01 % BHT and stored as in section 2.3.3. The coefficent of variation for this method was determined by repeated analysis of one diet (n=12 ; 40% energy fat, P/S 0.3) and a value of 1.96 % was calculated for 18:2(n-6).

2.3.7 Gas liquid chromatography.

The GLC system used was a Pye Unicam Series 204 gas chromatograph fitted with a PU4700 autoinjector and linked to a Trilab Model II integrator (Trivector). A glass column (1.5 m long , I.D. 2 mm) packed with a stationary phase of 10% SP2330 on 100/120 mesh Chromosorb WHW (Supelco) was used for all separations.

The following parameters were used :

injector temperature - 220°C

detector temperature - 300°C

column temperature - 180°C

hydrogen gas flow - 50 ml/min

carrier gas flow - 50 ml/min

air flow - 550 ml/min

Temperature programme

inital minutes - 3

rate °C/min - 3

final temp - 250°C

final minutes - 5

All fatty acids methyl ester peaks had been previously identified using authentic materials (Sigma standards ; 20:3(n-9) from Dr. EAM de Deckere, Unilever Research, Vlaardingen, The Netherlands and/or argentation chromatography (courtesy of Karin Lyall Cardiovascular Research Unit, Edinburgh)). The relative retention times with respect to C17:0 methyl esters were used thereafter.

Solutions of methyl esters stored in the freezer were equilibrated to room temperature before being placed on the autoinjector. Methyl esters from samples of PI and PS (5 ul) were injected on to the column using a 10 ul syringe, all other methyl ester samples were injected (1 ul) using a 1 ul syringe.

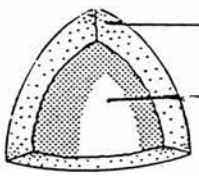
All results were stored on the Trilab system. After inital visual inspection for correct separation, identification and baseline tracking the samples were re-analysed where neccessary. All results were then transferred to a BBC computer disc and statistical analysis was carried out as described in section 2.5.

2.3.8 Prostacyclin measurement.

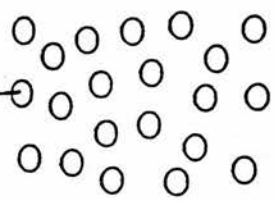
Prostacyclin was measured as its metabolite 6-oxo-$F_{1\alpha}$ using a radioimmunoassay involving a novel iodinated label and magnetic separation as described by Kelly [100]. Like all basic immunoassay systems this method has 4 major components; the labelled antigen, the antibody, the separation system and the unlabelled antigen (the sample). This method is novel with respect to the production of the labelled antigen and the separation system employed (Figure 2.4)

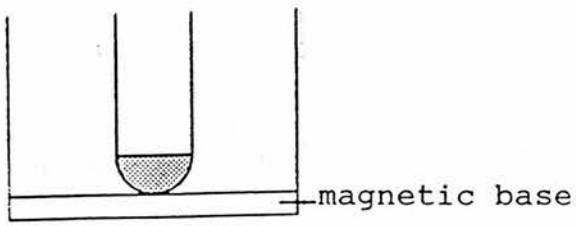
The labelled antigen was produced in response to an injection of methyloximated 6-oxo $F_{1\alpha}$. Methyl-oximation ensures production of a high sensitivity antiserum because endogenous ligands are unable to saturate the reaction. Furthermore, the required methy-loximation of all samples confers stability during storage and throughout the assay proceedure. The antigen is then coupled to ^{125}I label through an imide linkage rather than the conventional amide linkage thus confering further sensitivity.

FIGURE 2.4 - Amerlex



Each assay tube contains sev million particles in suspension. The Amerlex-M second Ab binds the primary Ab and hence permits separation of free and bound ligand.





After incubation, the assay tube is placed in contact with a magnetic base. The supernatant is decanted leaving dense pellet.

The use of Amerlex (Figure 2.4) reduces the time per assay as it combines antibody addition and separation to a single step.

The label, standards and antisera were all obtained from Dr Kelly (Centre for Reproductive Biology, Edinburgh). Amerlex (cat no. RPN 510) and magnetic packs were purchased from Amersham and all other chemicals from Sigma.

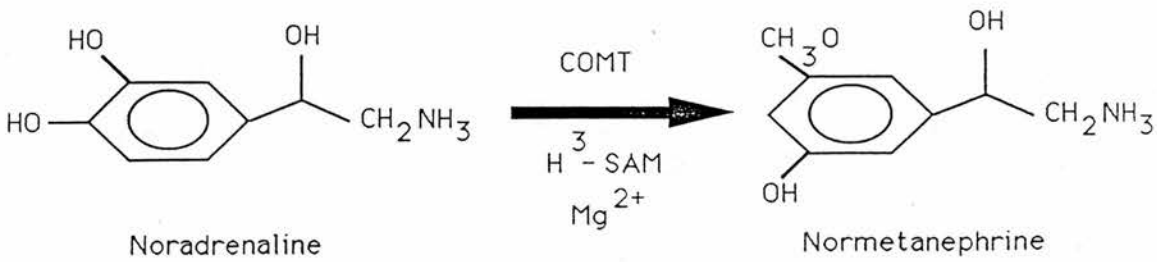
All samples were collected and stored according to the method described by Kelly [132]. The perfusate samples were assayed as described for PGE_2 measurement of tissue samples in the paper by Kelly et al [100]. All samples were measured in duplicate and quality control was prepared (perfusate plus standard) which gave an interassay coefficent of variation of 12 %. All results were expressed in ng / minute / gram wet weight of heart tissue.

2.3.9 Radioenzymatic measurement of noradrenaline.

Noradrenaline concentration in perfusate was measured radioenzymatically by Margaret Millar (Cardiovascular Research Unit, Edinburgh).

Noradrenaline was converted to its 3-0-methylated derivative using catechol-O-methyl transferase (COMT) in the presence of a radioactive methyl donor, ^3H - methyl-5-adenosyl methionine (^3H-SAM ; Figure 2.5).

FIGURE 2.5 Reaction involved in the radioenzymatic measurement of noradrenaline.



Noradrenaline Normetanephrine

The product (normetanephrine) was purified by selective ion pair extraction with tetraphenylborate, separated by TLC and subsequently oxidised to vanillin. The final product was counted in a liquid scintillation counter.

All chemicals were obtained from BDH and the standards were ordered from Sigma. The COMT was prepared as described by Da Prada and Zurcher [132] and was stable for at least three months when stored at -20°C.

500 ul of perfusate, collected on reperfusion was immediately placed in a Beckman Tube with 500 ul of 0.6N perchloric acid and stored in the -40°C freezer until assayed. The procedure for plasma samples as stated by Da Prada [132] was followed, except that the concentration of standards used was 60 and 600 fmole/tube. The coefficent of variation was calculated from the repeated analysis of the standards in one of

the experiments and gave a value of 12.4 % for the highest standard (600 fmol/tube) and 52.7 % for the lowest standard (6 fmol/tube).

All results are presented as pmol produced/gram wet weight heart tissue/minute.

2.4 - Langendorff perfusion.

2.4.1 Procedure.

The perfusion system adapted from Langendorff [134] was used (Figure 2.6). A modified Ringer-Locke solution (perfusate) was freshly prepared using double distilled water for each experiment. The ionic concentrations are shown in Table 2.6. The energy substrate used was glucose (5.5mM). The perfusate was maintained at 37°C and gassed continuously with 95% O_2, 5% CO_2 (2 l/min). Typical pCO_2, pO_2 and pH values are shown in Table 2.6.

Rats were anaesthetised with Sodium Pentobarbitone (60 ug/g body weight) by intraperitoneal injection. Heparin (0.25 units/g body weight) was injected into the femoral vein, a minimum of 30 seconds prior to the removal of the heart (this ensured an adequate distribution throughout the blood pool). The heart was then excised rapidly and placed in ice cold perfusate.

FIGURE 2.6 Experimental set-up for Langendorff perfusions.

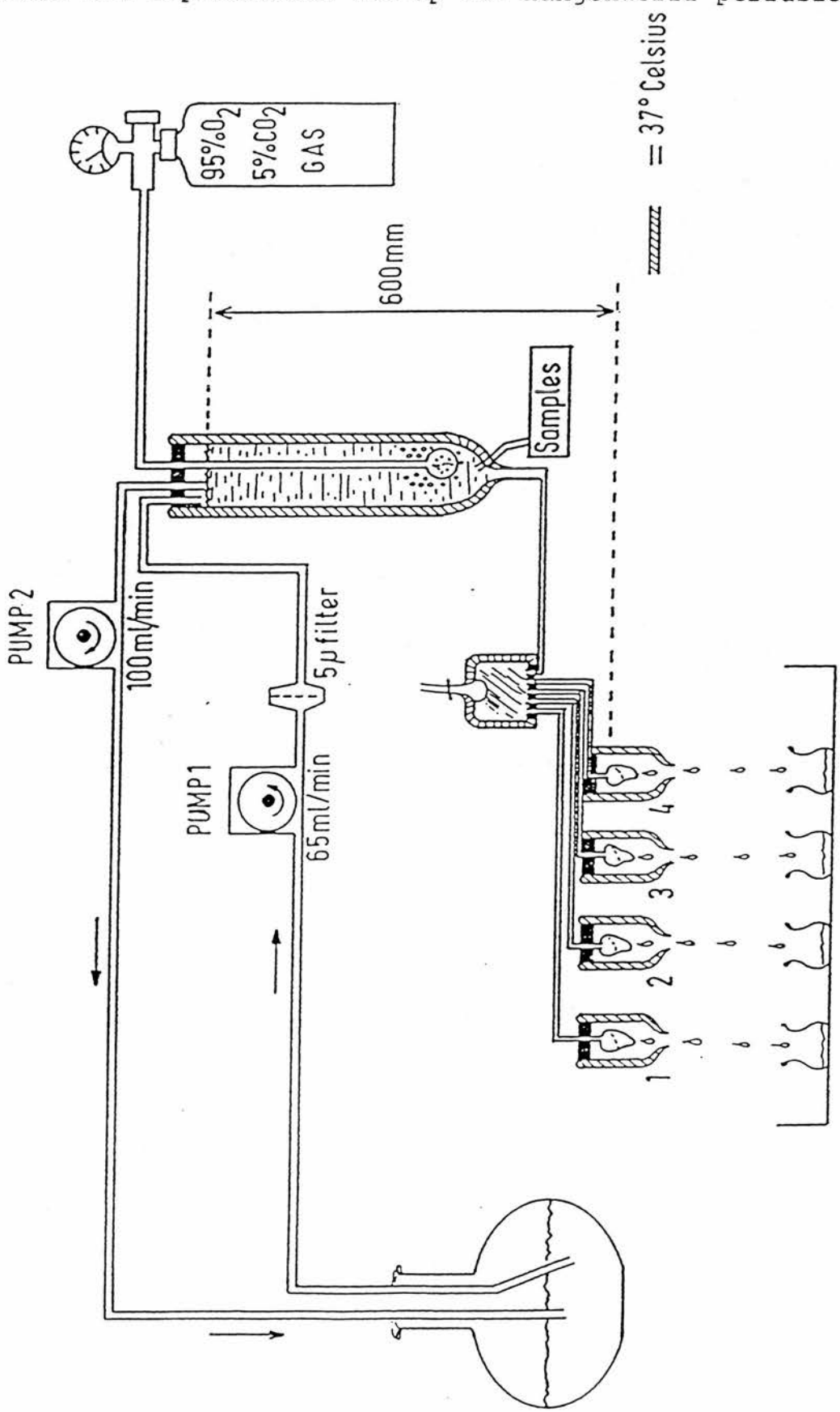
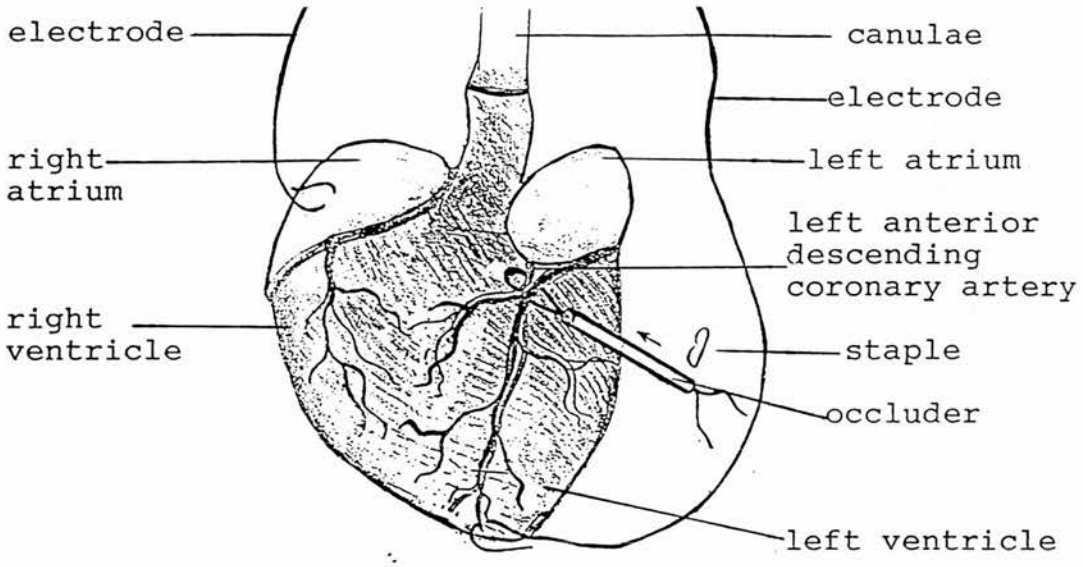


TABLE 2.6 - Ionic concentrations in the 'Perfusate'

Ion	Concentration
K^+	3.0mM
Ca^{2+}	2.5mM
Na^+	141mM
Cl^-	126mM
Mg^{2+}	1.0mM
PO_4^{2-}	0.4mM
HCO_3^-	25mM
pCO_2	35mmHg
pO_2	610mmHg
pH	7.39

The aorta was located and the heart was attached to the cannula using a 2/0 Mersilk thread (Figure 2.7). Four hearts were mounted per experiment (Figure 2.6). A ligature (Mersilk 4/0 16 mm suture) was placed around the left anterior descending coronary artery of each heart. Portex non-sterile polythene tubing (I.D. 0.86 mm ref 800/110/260/100) was then threaded through (Figure 2.7). This allowed occlusion and reperfusion of the coronary artery in each heart. Two silver ECG electrodes were then attached to each heart, one to the right atrium and the other to the anterior wall of the apex. The hearts were then left to stabilise for 5 minutes.

FIGURE 2.7 - Occlusion of the coronary artery



Occlusion of the artery was achieved by pulling the suture threads through the tube and securing them by clamping the tube with a staple (Figure 2.7).

The electrocardiograms were recorded continuously on a Gould TA 2000 recorder with a paper speed of 2.5 cm/s. After 20 minutes the Portex tubing was cut, thereby releasing the occlusion without damaging the heart. After 5 further minutes of reperfusion the experiment was completed. The hearts were removed, atria trimmed and then weighed and stored for possible analysis.

2.4.2 Protocol.

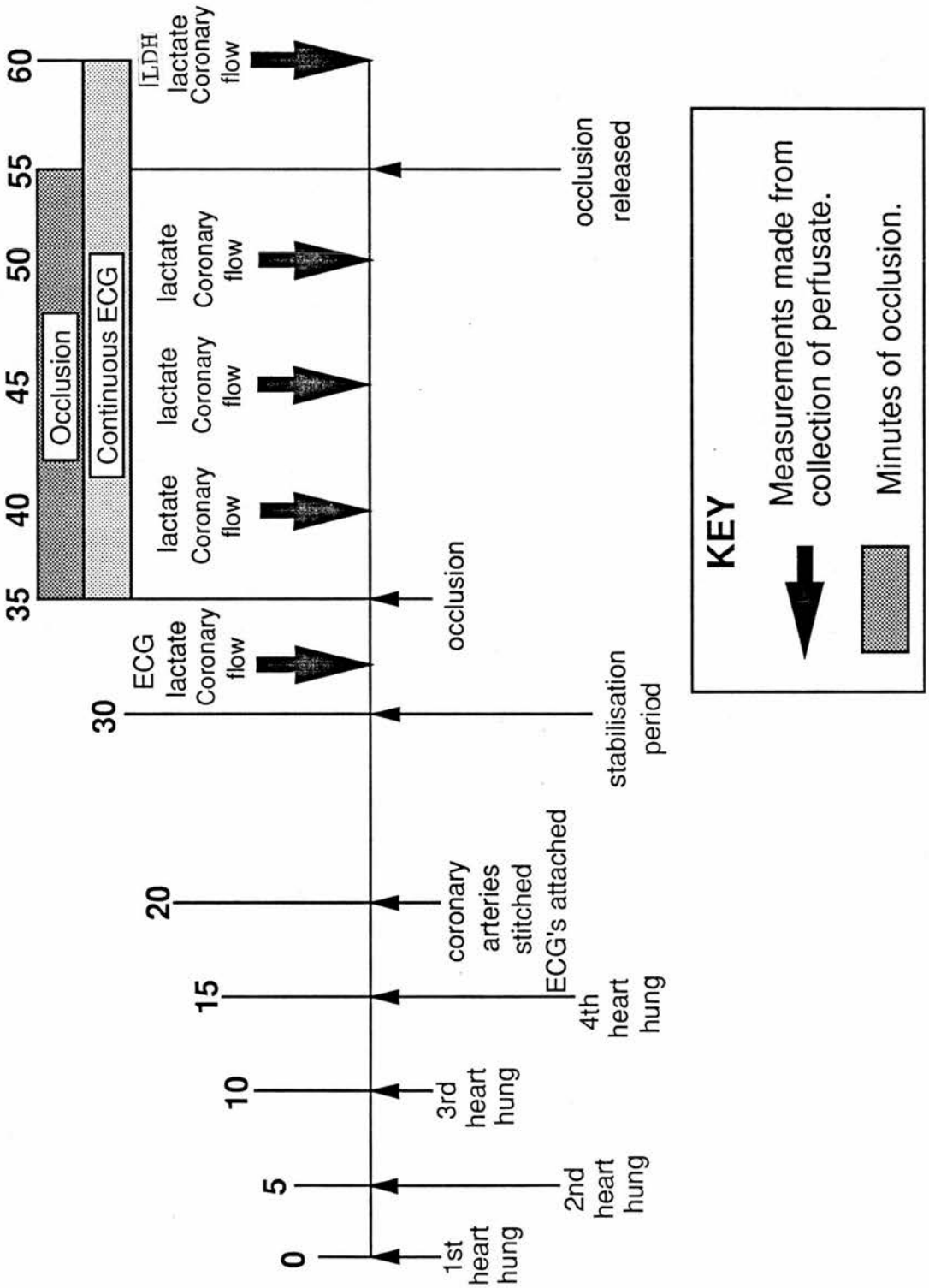
A basic protocol for each perfusion experiment was applied. All arrhythmias were screened using the

Langendorff perfused isolated male rat heart model (Figure 2.6). The hearts were all studied when the animals were 16 weeks of age without prior knowledge of their dietary status. All experiments were designed with a contemporary control group. The group size was calculated using the required statistical equation (see Section 2.5.1) as stated in the Lambeth convention [109]. The presence of arrhythmias was monitored for 20 minutes of ischaemia and during 5 minutes of reperfusion. Figure 2.8 shows the measurements which were taken in each experiment to monitor the degree of occlusion and subsequent ischaemia. Coronary flow was calculated by weighing the amount of perfusate collected from the heart over a 30 second time interval. The methods for all the other measurements are described in section 2.3.

Adipose tissue samples were taken for triglyceride fatty acid analysis from the inguinal region of each rat from every fourth run (1 run = 1 complete experiment as in Figure 2.8). The ventricles of the hearts from every fourth run were stored in liquid N_2 for phospholipid fatty acid analysis.

The electrocardiograms were analysed 'blindly' for the arrhythmia parameters VF, VT and ectopic beats, classified as stated in the Lambeth Convention [109] (see Figure 1.9 for some examples of classification used).

FIGURE 2.8 Experimental protocol for arrhythmia screening on the Langendorff in-vitro perfusion system.



The heart rate was monitored pre-occlusion, 5 and 10 minutes after occlusion, but not at 15 or 20 minutes because the presence of arrhythmias over this time period made measurement inaccurate.

Results for a particular heart were excluded if there were irregularities in the pre-occlusion electrocardiogram and if two or more of the following criteria occured:

(1) coronary flow reduction was less than 30 %

(2) initial lactate concentration was greater than 1 uMoles produced/min/g wet weight of heart.

(3) reperfusion lactate concentration was less than 1 uMoles produced/min/g wet weight of heart.

(4) LDH concentration was less than 0.5 Units/min/g wet weight of heart.

All the remaining results were then analysed using the statistical methods described in the next section.

2.5 - Statisitical analysis.

All statistical tests were carried out using the Minitab statistics package. To ensure non-bias analysis, a statistical protocol for each parameter was defined before the start of all experimental analyses. The tests used and the parameters they were applied to are described in the following sections.

2.5.1 Design of experiment.

The group size for experiments necessary to demonstrate a 50 % reduction in the incidence of VF ($p<0.05$) was calculated using the 'sample size' equation for unequal groups [135], using the incidence of the control group to define the 'normal' population (Chapter 3 discusses this topic further).

2.5.2 Statistical protocol for results from perfusion experiments.

All biochemical measurements were normally distributed and the statistical protocol was one-way analysis of variance of all the data. Follow up analysis was carried out using an unpaired t-test on the parameters which were significantly different ($p < 0.05$).

Arrhythmia incidences are classed as frequencies and were therefore analysed using the chi-square test. An overall chisquared test was applied and if a significance $p < 0.05$ was reached, then individual chi-square tests were carried out to examine which groups differed.

The other arrhythmia parameters such as onset and duration had skewed distributions and were analysed using a non-parametric test (Mann Whitney). Values $p < 0.05$ were accepted as significant.

2.5.3 Statistical protocol for the analysis of fatty acid results.

All results were analysed using an identical protocol for a normally distributed population, as described by other experimentalists [136] [82]. Fatty acid composition, expressed as a percentage of the total weight, can represent problems due to the inter-dependance of the relative amounts of the individual fatty acids. All results for myocardial fatty acids were therefore expressed as absolute amounts. Adipose tissue was still expressed as a percentage of the total fatty acids because it was only used as a general indicator of diet. One-way analysis of variance was carried out on all the fatty acid data from each experiment, following which an unpaired t-test was applied to the parameters which were statistically significant to determine which groups differed. To assess the biological importance of these changes with respect to VF, correlations were made between the incidence of VF and the mean value of any fatty acids which were statistically significantly different.

2.5.4 Miscellaneous.

All other parameters were analysed using one way analysis of variance followed by an unpaired t-test when $p<0.05$ was reached.

All results are presented as the mean and standard deviation.

CHAPTER 3

IDENTIFICATION OF AN INBRED STRAIN OF RAT WITH A HIGH INCIDENCE OF VF.

3.1 - Introduction.

The validity of all experiments depend on their experimental design. The use of contemporary controls throughout the course of the thesis was necessary as there was no previous information on the incidence of VF in animals fed semi-synthetic diets. This chapter deals with some of the statistical considerations required when testing an antiarrhythmic effect. The occurence of serious ventricular arrhythmias is of undisputed clinical importance, but it is a weak statistical end-point. Often experimental studies give little thought to the group sizes required to test an antiarrhythmic effect. The numbers can sometimes be too small and it is not uncommon for results of anti-arrhythmic interventions to be published on group sizes of 10 or less [40] [118] [125]. In contrast to laboratory experiments, these problems are well recognised in clinical trials and can be overcome by the calculation of the correct group size.

The correct group size can only be calculated apriori if the expected incidence of arrhythmias in the population (ie controls) is known. Many experimentalists do not know the expected incidence and cannot, therefore, define the necessary group sizes. The minimum group size required to demonstrate a specific change in the incidence of VF can be derived from Maisland contingency tables [137] (Figure 3.1) or from

the 'group size' equation [135] (Figure 3.2), depending on the particular requirements.

FIGURE 3.2 - Equation to calculate 'sample size' assuming final populations could be unequal.

$$\text{Sample number per group} = \frac{k\,(\,var_1 + var_2)}{D^2}$$

var_1 = 100 - incidence in group 1 (%)

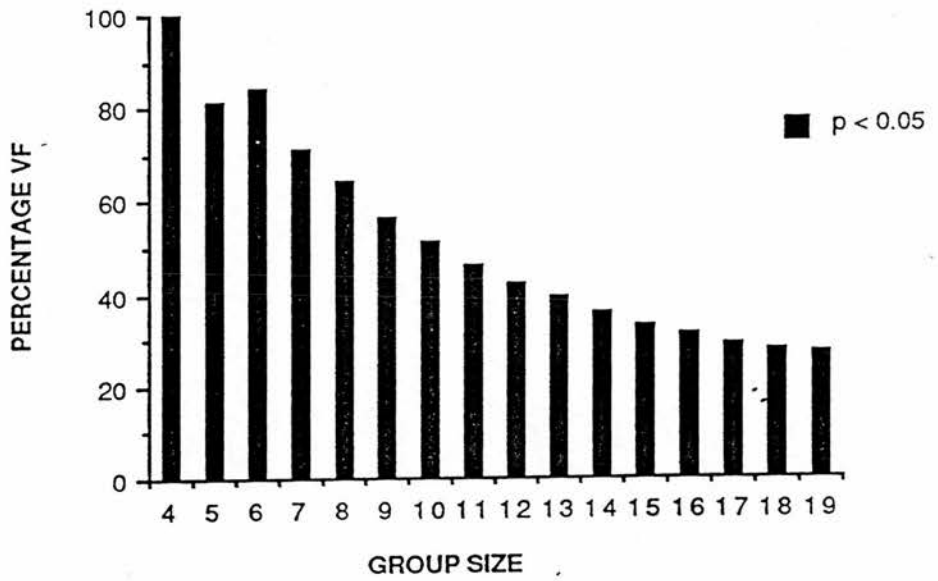
var_2 = 100 - incidence in group 2 (%)

D = difference to be detected

k = constant which depends on the significance level and power required (e.g. 1% or 5%)

Previous work carried out by Riemersma and Saman [41] showed an incidence of VF during coronary ligation in the isolated perfused heart from Sprague Dawley rats fed 'control' diet (see section 3.2) to be 30%. Assuming a 50% reduction in the incidence of VF to be clinically relevant then the minimum group size required for an experiment to show this effect with a power of 90% at a 5% level of significance (using the equation in Figure 3.2) was 80. These very large numbers imposed limitations on the number of experiments which could be completed due to time and money. If the basal incidence of VF was greater, then the group sizes would be smaller.

FIGURE 3.1 Minimum Group Requirements to document a difference in the incidence of VF



Ordinate indicates detectable percentage reduction from control, for a given group of equal size at a significance level of $p<0.05$.

Therefore, the aim of this first experiment was to screen inbred rats to isolate a strain which had a high incidence of VF under standard experimental and dietary conditions. Inbred rats were chosen to reduce the genetic variation expected with an outbred population.

Adipose tissue biopsies were taken to monitor the animal's dietary intake. Heart tissue samples were also taken to study the possible involvement of changes in myocardial phospholipid fatty acids with the incidence of ischaemic VF.

3.2 - Methods.

7 inbred strains of rat were selected on the basis of availability and cost (Table 3.1). The outbred Sprague Dawley Rat was selected as a positive control as its incidence of VF on "control" diet had been studied previously [41].

The 'control' diet chosen for all the studies, was the diet consumed by the average Scottish male : 40% energy fat with a P/S ratio of 0.3 [62]. This diet was chosen as one of the major aims of this thesis was to examine the effects of diets which could be consumed by the average man on experimental animals.

The diet was calculated and made as described in section 2.2 (ingredients listed in Appendix 3).

TABLE 3.1 - Inbred strains of rat.

Strain	Supplier
Hunter	MRC Unit, Edinburgh University
Hypertensive	MRC Unit, Edinburgh University
Obese	Rowett Institute, Aberdeen
WKY-CR	Charles River, Margate
Spontaneous Hypertensive (SHR)	Charles River, Margate
F344	Harlan Olac, Oxon
Lew	Harlan Olac, Oxon

15 male rats from each strain were placed on the "control" diet at 8 weeks of age. The animals were housed and cared for as section 2.1. After 8 weeks of feeding they were anaesthetised, the hearts isolated and screened for their incidence of VF during acute experimental ischaemia using the standard methods and protocol described in section 2.4. Adipose and heart tissue were taken and analysed as described in section 2.3.1 - 2.3.2 for triglyceride and phospholipid fatty acid analysis.

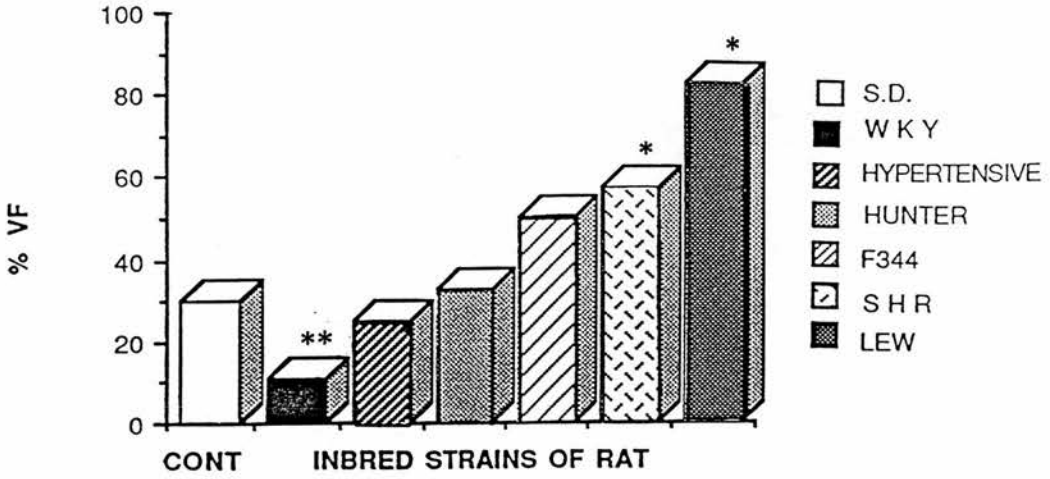
3.3 - Results.

3.3.1 Arrhythmias.

There were marked statistically significant differences in the incidence of VF between the strains ($p<0.01$; Figure 3.3). Lew rats had the highest incidence and therefore this strain was chosen for future experiments. The obese strain of rat was omitted from all the following results because of the high incidence of preocclusion arrhythmias in all but 3 animals, the final number being too small to use in any statistical comparisons.

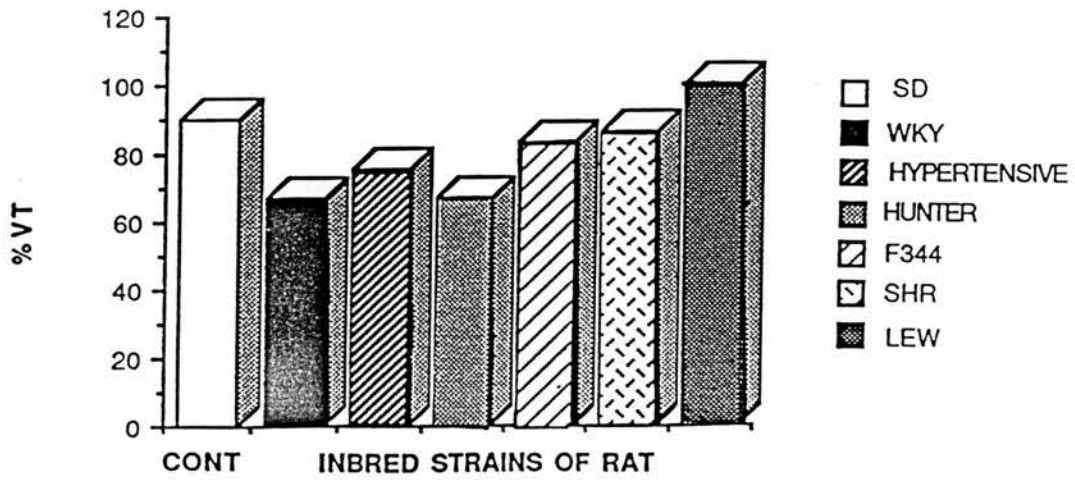
The incidence of VT was much higher than that of VF and, although a similar trend as seen with VF was observed (Figure 3.3), statistical significance was not reached (Figure 3.4).

FIGURE 3.3 - Incidence of VF during acute coronary ligation in isolated perfused hearts from different inbred strains of rat.



*p<0.01 (Chi-square test)

FIGURE 3.4 - Incidence of VT during acute coronary ligation in isolated perfused hearts from different inbred strains of rat.



N.S. after Chi-square test

There was a trend towards an earlier onset and a longer duration of VF with increasing incidence of VF (Table 3.2); however these results were not statistically significant.

TABLE 3.2 - Time to onset and duration of VF during acute coronary artery ligation in isolated hearts of inbred strains of rat.

Strain	Onset VF (minutes)	Duration VF (minutes)
WKY	10.52 (7.00)	2.96 (3.97)
HYPERTENSIVE	12.91 (3.42)	3.57 (1.93)
HUNTER	13.16 (6.36)	2.51 (2.94)
F344	9.57 (1.81)	3.38 (4.34)
SHR	10.17 (2.55)	3.99 (4.92)
LEW	9.39 (3.74)	3.76 (5.77)

All values are mean $\pm$ S.D. ; N.S. (Mann-Whitney test).

Reperfusion VF as distinct from ischaemic VF was not different between the strains, with an average incidence of 90% (not shown).

The results in Figure 3.3 and 3.4 were not due to variations in the ischaemic zone, as the absolute and relative reduction in coronary flow were not signif-

icantly different between the strains.

3.3.2 Myocardial phospholipid levels.

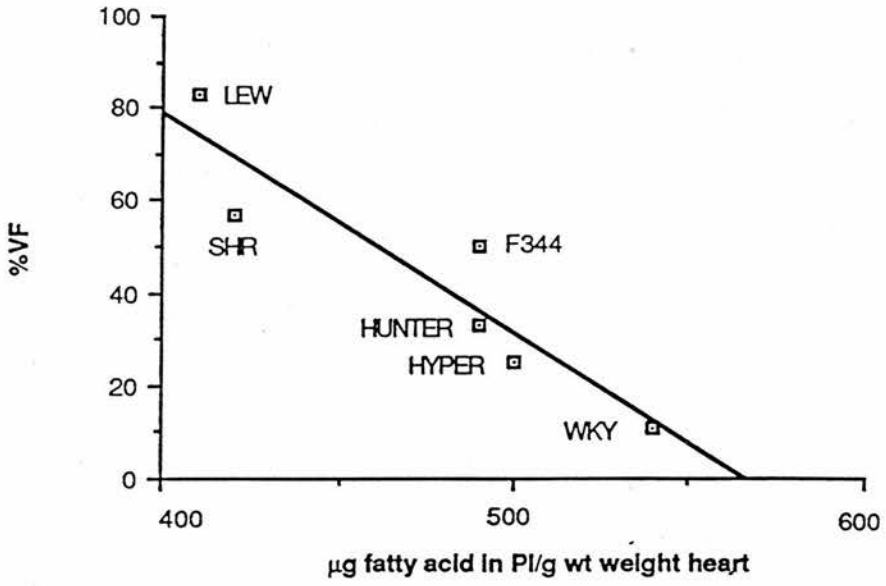
Total phospholipid, PC, PE and PS levels did not differ between the strains (Table 3.3). The amount of fatty acids present as PI and CL phospholipid classes did, however, differ between the strains (Table 3.3). An inverse relation was observed between the myocardial PI level and the incidence of VF in the inbred strains ($r=-0.920$; Fig 3.5). There was a difference in CL levels between the various strains. However, this difference did not correlate with the incidence of VF, although it was higher in the hypertensive animals possibly reflecting an increased density of mitochondria in these animals.

TABLE 3.3 - Total and individual phospholipid Levels (ug fatty acid/g wet weight) in hearts from inbred rats

Phospholipid	Inbred Strain					
	WKY	HYPER	HUNTER	F344	SHR	LEW
TPL	10620 (820)	9854 (767)	9771 (1155)	10168 (780)	9704 (188)	9800 (852)
PI	545$^{\#o}$ (58)	510 (66)	471 (77)	496 (55)	404$^{\#}$ (79)	420^{o} (104)
PS	236 (53)	204 (41)	322 (86)	202 (38)	312 (84)	249 (111)
PC	4991 (445)	4982 (603)	4918 (963)	4689 (754)	4711 (441)	4754 (493)
PE	3862 (278)	3519 (232)	3535 (431)	3607 (384)	3423 (478)	3237 (804)
CL	2022$^{+/}$ (253)	1961$^{*\$}$ (172)	1640 (342)	1688^{*+} (190)	1750 (201)	1688$^{\$/}$ (230)

Identical symbols indicate statistical significance between the 2 groups in the horizontal plane ; $p<0.05$.

FIGURE 3.5 Correlation between the amount of fatty acids in PI and the incidence of VF.



3.3.3 Myocardial phospholipid fatty acid composition

The absolute amounts of the 17 individual fatty acids of the 5 phospholipid fractions showed numerous differences. In order to assess the possible physiological importance of such differences with respect to the development of VF further statistical analysis was carried out. The fatty acids of the individual phospholipid classes which were significantly different between the 6 strains were related with the incidence of VF. Seven fatty acids were significantly correlated ($p<0.1$) with the incidence of VF (Table 3.4).

TABLE 3.4 Correlations between the statistically significant fatty acids in myocardial phospholipid and incidence of VF.

Phospholipid Fraction	Fatty Acid	R value	P value
PI	20:3(n-9)	-0.858	< 0.01
PC	18:1(n-9)	-0.729	< 0.05
PE	18:2(n-6)	-0.674	< 0.1
PE	20:3(n-6)	-0.723	< 0.05
PE	20:4(n-6)	-0.600	< 0.1
CL	18:2(n-6)	-0.697	< 0.1
CL	20:4(n-6)	0.622	< 0.1

3.3.4 Fatty acid composition of adipose tissue.

All measured fatty acids in adipose tissue differed between the 6 rat strains except 20:1 (n-9) and 22:6 (n-3) (Table 3.5).

The P/S ratio was lower in the SHR strain in comparison to other inbred strains ($p<0.05$; Table 3.3). This is due to a reduction in PUFA, primarily linoleic acid.

Low dietary linoleic acid, which is associated with low levels in adipose tissue may lead to serious ventricular arrhythmias in rats [138]. However, no correlation was seen with linoleic acid in this experiment, although both 16:0 and 20:3(n-6) correlated with VF (r= 0.738 and -0.699 respectively).

TABLE 3.5 Adipose tissue fatty acids in different rat strains which were statistically significant ($p<0.05$).

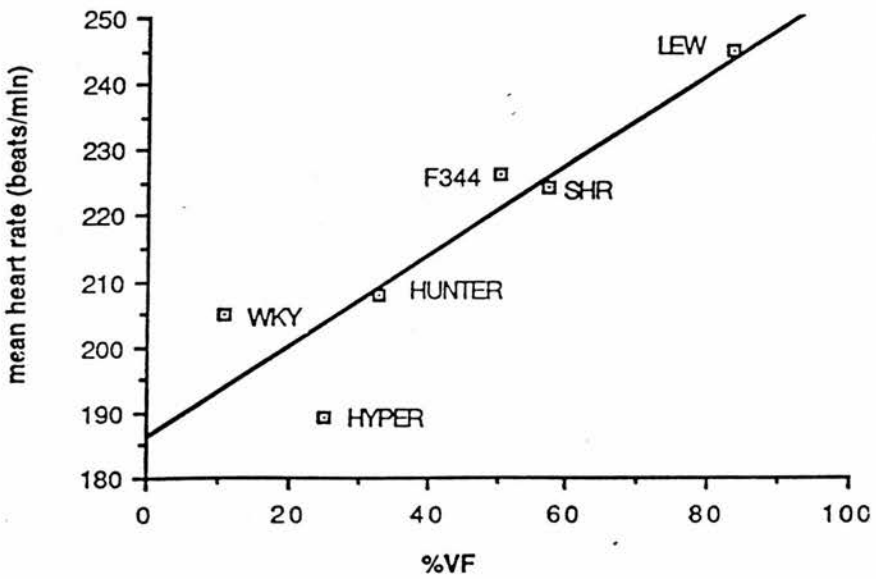
(Units = percentage of total fatty acids)

FATTY ACID	INBRED STRAIN					
	WKY	HYPER	HUNTER	F344	SHR	LEW
Saturated						
16:0	21.63 (2.05)	20.64 (1.22)	21.59 (.70)	23.80 (1.45)	23.87 (1.26)	23.23 (1.52)
18:0	8.44 (1.72)	9.16 (1.10)	5.39 (.91)	7.16 (.95)	7.57 (.92)	6.54 (.58)
Monounsaturated						
16:1	4.77 (1.73)	3.86 (.55)	6.74 (.87)	5.17 (.95)	5.50 (.73)	5.52 (.58)
Polyunsaturated						
(i) (n-3) class						
22:5	.052 (.024)	.029 (.027)	.029 (.012)	.035 (.012)	.049 (.012)	.037 (.012)
(ii) (n-6) class						
18:2	14.30 (1.45)	15.15 (1.20)	15.15 (1.13)	14.83 (0.97)	12.68 (1.53)	15.24 (0.96)
20:3	.051 (.014)	.036 (.018)	.032 (.009)	.012 (.006)	.039 (.009)	.018 (.019)
20:4	.444 (.136)	.387 (.277)	.248 (.071)	.390 (.124)	.438 (.083)	.389 (.149)
22:4	.140 (.067)	.181 (.173)	.058 (.021)	.112 (.061)	.106 (.027)	.089 (.070)
sats	30.12	29.85	26.99	30.96	31.48	29.77
pufa	15.10	15.90	15.60	15.47	13.42	15.88
mono	52.42	51.93	54.73	52.99	52.63	54.25
P/S	0.50 (.06)	0.53 (.03)	0.58 (.03)	0.50 (.04)	0.43 (.07)	0.54 (.06)

3.3.5 Heart rate.

Heart rate over the first 9 minutes of occlusion depended on the strain studied ($F = 24.50$; $p<0.001$) and, to a lesser degree on the time after coronary artery ligation ($F = 4.09$; $p<0.05$). A trend was observed for increasing heart rate with increased incidence of VF. A correlation was found between mean overall heart rate and the incidence of VF ($r=0.899$; $p<0.01$; Figure 3.6)

FIGURE 3.6. - Correlation of the overall heart rates of different inbred rats and their incidence of VF



3.3.6 Measurements to assess ischaemia.

Statistically significant differences were seen in the initial, 15 min occlusion and reperfusion

coronary flows between the different strains (Table 3.6). The differences in flow rate (expressed as ml/min/g heart weight) were the result of the increased heart:body weight ratios of the MRC hypertensive strain and the SHR strain.

TABLE 3.6 - Statistically significant differences in in coronary flow.

Strain	Coronary flow		
	Initial	15 min	Reperfusion
	(ml/min/g)		
WKY	9.03^{b} (4.91)	5.61^{d} (5.08)	7.25^{bd} (4.52)
Hypertensive	6.21^{c} (2.15)	3.49^{ac} (1.14)	4.08^{abeg} (1.21)
Hunter	7.93^{a} (2.45)	5.68^{ab} (2.40)	6.23^{ac} (2.15)
F344	9.56^{cd} (3.11)	5.35^{d} (3.27)	6.25^{ef} (2.60)
SHR	5.36^{abde} (1.19)	2.54^{bce} (0.98)	3.36^{cdfgh} (1.72)
Lew	7.48^{e} (2.39)	4.52^{e} (1.89)	6.28^{h} (2.51)

Identical symbols indicate significance between the 2 groups in the vertical plane after unpaired t-test ; $P<0.05$.

3.3.7 Body and heart weights.

Final body and heart weights showed significant differences between strains (Table 3.7).

Heart weight is known to be correlated with body

weight [139] and this was confirmed. However, the SHR and MRC hypertensive strains both have enlarged hearts, due to their expected hypertrophy, and are the exceptions.

TABLE 3.7 Final body weights, heart weight and heart:body weight ratios.

STRAIN	BODY WEIGHT (Grams)	HEART WEIGHT (Grams)	HEART:BODY (Grams)
WKY	308[a] (41.31)	1.007[a] (0.121)	3.27[a,b,c,d] (0.16)
HYPER-TENSIVE	323 (42.45)	1.305[b] (0.325)	4.02[e,f,g] (0.79)
HUNTER	270[b,c] (65.56)	0.808[b,c] (0.197)	3.01[e,h,i,j] (0.30)
F344	339[d,e] (24.16)	0.910[d,e] (0.055)	2.69[c,j,k] (0.17)
SHR	306[d,f] (15.56)	1.158[a,c,e] (0.069)	3.79[b,i,k,l,m] (0.29)
LEW	371[a,c,e,f] (15.67)	1.048[d] (0.087)	2.82[g,d,m] (0.16)
OBESE	431[b] (34.07)	1.018 (0.130)	2.36[h,e,a,i] (0.17)
ANOVA[#]	F=14.35	F=11.05	F=26.23

Identical symbols indicate statistical significance between the 2 groups in the vertical plane after unpaired t-test ; $p<0.05$.

Overall statistical analysis of variance

3.4 - Discussion.

Clinical studies have identified genetic predisposition to sudden cardiac death in the long QT syndrome and hypertrophic cardiomyopathy (HOCUM) [140] [141]. To date no experiments have been designed to study the genetic influence on sudden death during acute myocardial ischaemia. This is the first report to suggest a genetic influence on the incidence of VF during acute myocardial ischaemia in different strains of animals within one species. The results in Figure 3.3 show that there was a variation in the incidence of VF in the inbred rats screened. The high incidence of VF in the Lew strain allowed a reduction of the minimum group size from 80 to 32 in our particular experimental design and consequently more dietary interventions could be studied. The choice of strain and not simply the species is therefore an important consideration when designing a study.

No significant differences were seen in the incidence of VT or reperfusion VF. From a statistical point of view it could be argued that the group size was too small to show a significant decrease in VT. Therefore, an increase in numbers could show a similar statistically significant variation for VT, as observed with VF. The incidence of reperfusion VF in this model was unaffected in different strains, possibly indicating that the mechanism for reperfusion VF differs from

ischaemic VF as has been reported by other researchers [142]. The very high mean incidence of reperfusion VF could be a result of the reduction in K^+ (from 5.6mM to 3.1mM) and/or a result of the semi-synthetic high fat diet. A trend was also observed towards an increased duration and earlier onset with an increase in VF incidence. The very high standard deviations for the onset and duration of VF suggests greater numbers would be required for full statistical analysis of these particular parameters.

Experimental evidence suggests that membrane phospholipids could be involved via three different mechanisms, either individually or in combination. First, a relationship was observed between the relative amount of PI and the incidence of VF ($r=-0.920$). PI is the precursor of PIP_2 which generates IP_3, a second messenger, which is involved in the control of Ca^{2+} homeostasis in the cell [143]. The results suggest an increased hydrolysis of PI and its phosphorylated products by phospholipase C during ischaemia. This is supported by a recent paper which reported accumulations of radiolabelled inositol monophosphate, inositol bisphosphate and inositol triphosphate in reperfusion fluid after 30 minutes of global ischaemia [144]. This situation could lead to an influx of Ca^{2+} into the cell and thereby induce VF. Ca^{2+} influx is generally recognised as arrhythmogenic

[145] lending further support to this particular hypothesis. Further experiments to determine the effect of coronary ligation on PI turnover, PI metabolites and Ca^{2+} would establish a greater understanding of the possible involvement of this mechanism.

The experimental results here also indicate phospholipid compositional changes which may alter prostanoid production and hence the incidence of ischaemic VF. Prostanoids have been reported by several workers to be involved in an antiarrhythmic mechanism [71] [146] [147]. PGI_2 and the synthetic analogue iloprost have been identified as antiarrhythmic in the isolated perfused heart [146] [148]. The results in this chapter suggest a possible involvement of 20:4(n-6) in PE ($r = -0.6$) and 20:4(n-6) in CL ($r = 0.622$) in the genesis of VF. The arachidonic acid in CL only represents 3% of the total fatty acids in the heart whereas in PE it accounts for 28%. CL is found predominantly in the mitochondrial membrane [149] and its relation to prostanoid synthesis is obscure. In contrast, the changes in arachidonic acid in the PE fraction could be of greater importance for prostanoid synthesis in the sarcolemmal membrane. Increased incorporation of arachidonic acid into PE might increase the precursor available for prostanoid synthesis, which in turn increases prostanoid production. PGI_2 is the major prostanoid secreted by the isolated perfused heart

[150] and Coker et al have shown PGI_2 to be anti-arrhythmic [146]. Therefore, changes in arachidonic acid levels in PE may be the mechanism by which the genetic variation in VF is controlled. Measurement of prostanoids and monitoring the effects of prostanoid inhibitors would have provided more information and this will be examined in Chapter 6.

Changes in the composition of other PUFA's in myocardial phospholipids also correlated with the incidence of VF and suggested the possible involvement of yet another factor (Table 3.4). As early as 1930 the n-6 fatty acids were discovered to be essential for several biological functions, including maintenance of normal membrane function [52]. As yet, the specific role of the n-6 PUFA in membranes has still to be established. The n-6 PUFA's show major compositional changes in the present study as well as 20:3(n-9) in PI. The membranes of some of the rat strains could have been partially EFA deficient, causing alterations in membrane function and leading to VF, since 20:3(n-9) is considered an indicator for EFA deficiency [151]. However, the correlation with VF was negative, elevated 20:3(n-9) accompanied a decreased incidence of VF. Futhermore no external indicators of EFA deficiency were observed (ie scaly skin, loss of hair etc) and the animals were all fed the same diet which contained 3% energy from linoleic acid (sufficent to satisfy EFA

requirement). Increased levels of all the PUFA's identified correlated with decreased VF. As the fatty acids in question were all highly unsaturated, they could have exerted their effect directly by an increase in membrane fluidity [98]. No evidence exists to illustrate that an increase in fluidity alters the activity of transmembrane proteins in the myocardium, but it is an area of considerable study. The fatty acids in this experiment could have increased the number of functional ion channels or the activity of existing ion channels. To confirm the role of this particular hypothesis, specialised studies on the effect of PUFA'S on transmembrane function, either in-vivo or in-vitro, would have to be carried out.

Previous studies have identified a possible involvement of heart rate in ischaemic VF. Increased heart rate results in increased oxygen consumption and hence increased ischaemic damage and predisposition to VF [152]. Changes in heart rate may also predict VF by reflecting increased catecholamine release, which is assumed to lead to ischaemic arrhythmias [153]. It was, therefore, of interest that increased heart rate correlated with an increased incidence of VF. In the experimental model used here sympathetic nerves have been severed but catecholamine release can still be observed as a result of direct noradrenaline release from the nerve endings [154]. Direct measurements of catechol-

amine release and high energy nucleotides could validate this statement further. Heart rate in different mammals has been reported to correlate to the relative amount of 22:6(n-3) in myocardial total phospholipid and PE [155]. The data from this experiment showed a correlation ($p<0.1$) between 22:6(n-3) in PE and heart rate, but no relationship with its relative amount in total phospholipid. No correlation of 22:6(n-3) in PE with the incidence of VF was found suggesting this is probably not the mechanism responsible for the difference in the incidence of VF between the various rat strains.

Adipose tissue fatty acid composition has been shown to be an indicator of dietary fat intake [156]. In this experiment all the animals were fed the same diet, but statistically significant differences were still observed (Table 3.5). A selective mechanism for deposition of dietary fat adipose tissue has not been demonstrated. It is simply a storage depot for excess fat. Thus, these findings suggested either a selective process of fat absorption, processing in the liver, or an incomplete equilibration of dietary fatty acids with body adipose tissue. Changes in the absorption of specific triglycerides could lead to some fatty acids being incorporated into phospholipids in the liver and others being oxidised. A variation in the metabolic rate of the animal would alter the turnover

in adipose tissue, and the fatty acid pattern observed could be predominantly that of the previous dietary intake. Differing activities of delta-6 and delta-5 desaturase between strains would also effect the utlimate fate of linoleic acid. Therefore the relative amounts of fatty acids in adipose tissue, originally from endogenous and exogenous fatty acid sources, could be changed. Another possible effector could be the maternal diet, which was uncontrolled in these experiments. This has been identified as an important indicator in arrhythmia experiments in a collabortive experiment with Organon [157]. In this experiment enquiries to the animal suppliers involved in this study revealed that all animals had been bred on the same 'Labsure' diet suggesting this factor was not of great importance. However, analysis of Labsure diet over a 6 month period revealed that significant variations in the EFA's 18:2 (n-6) and 20:5 (n-3) occur (Table 3.8).

Genetic influences on a number of biochemical processes appear to be the major factor responsible for the composition of fatty acids in adipose tissue and perhaps the incidence of VF. However, further experiments would have to be carried out to elucidate the roles of the various effectors.

TABLE 3.8. - Variation in EFA composition of Labsure diet over 6 month period.

	MEAN (%)	S.D. (%)	C.V. (%)
18:2(n-6)	49.51	4.26	8.6
20:5(n-3)	1.61	.60	37.8

C.V. = coefficent of variation.

Interesting observations, secondary to the aim of this experiment, were made in the case of hypertension and its relationship to arrhythmias. Hypertrophy of the heart has been linked with increased cardiac damage during ischaemia [158] and an increased incidence of VF was expected. Indeed the incidence of VF in the SHR strain was higher than the WKY control strain. In contrast, the hypertensive strain from the MRC unit Edinburgh showed a significantly lower incidence of VF in comparison to the SHR rats, but no difference in VF when compared to the normotensive WKY. Thus, even in this case alternative, presumably genetic factors operate. Furthermore, the developmental stage of hypertension has been linked to vulnerability in the ischaemic situation [159]. But, heart to body weight ratios showed no difference between SHR and hypertensive rats suggesting this was not involved. These

results therefore identify the hypertensive and SHR as models of interest in the study of hypertrophy and its relationship to VF.

In conclusion, this experiment has identified a strain with a high incidence of VF fed a standardised 'control' diet. A number of mechanisms for the differences in the generation of VF have been proposed from the results of myocardial phospholipids and adipose tissue fatty acid analysis, as well as from other factors known to relate to VF. The major factor acting on the different strains, responsible for the variation in the incidence of VF appears to be the genetic control of dietary fat incorporation into myocardial phospholipids, which in turn may influence various phospholipid functions. This study was not designed to test these factors, but further experiments would be merited to clarify their importance.

CHAPTER 4

N-6 FATTY ACIDS AND VENTRICULAR FIBRILLATION.

4.1 Introduction.

Fats form an important constituent of mans diet and provide a considerable source of energy (~40% of total energy in Western industrialised societies [62]). An awareness has developed that excess dietary fat is a major risk factor for coronary heart disease in man. Simultaneously there is general agreement that saturated animal fat is detrimental to health and should be reduced. Accordingly the World Health Organisation has recommended an ideal dietary fat intake of 30% energy and a minimum P/S ratio of 1.0 [42].

Diets enriched in PUFA predominantly 18:2(n-6) having a high P/S ratio, may reduce the incidence of ischaemic ventricular fibrillation in animals [39] [40] [41] [72]. These studies have all used standard laboratory chow supplemented with fat, but the study of such diets poses several problems. Firstly, the high calorific content of the fat supplement results in a reduced intake of the basic chow which contains all essential vitamins and minerals. Secondly, the diets used were very extreme fat diets, which could not be consumed by man, e.g. a P/S ratio of 6.46 [39]. Consequently an effect on ischaemic VF cannot simply be attributed to the higher intake of polyunsaturated fatty acids. The content of vitamin E was not controlled and low levels of this vitamin have been linked with sudden death and VF [120]. The first aim of this chapter was

to use isocalorific diets to confirm or otherwise the possibility that the antiarrhythmic effect observed in the previous studies was indeed due to the increased content of polyunsaturated fatty acids. Furthermore, the diets to be tested would not be so extreme as to preclude extrapolation of the results to man. The diets chosen was the average diet consumed by Scottish men (40% energy fat P/S=0.3, control diet) and the achievable ideal (P/S ratio of 2.0 40% energy fat).

A reduction in total fat consumption, as recommended, would invariably result in a reduction of polyunsaturated fatty acids. Therefore the secondary aim of this chapter, was to examine whether the antiarrhythmic effect of a linoleic acid rich diet could be maintained in 'prudent' low fat diets. The relative amount of energy from fat of 40%, 30% and 20% total dietary energy was studied. These reductions may not appear large in numerical terms, but they correspond to a 25 and 50% reduction in the average Scottish man's daily dietary fat intake.

4.2 - Methods.

6 diets were calculated to give the energy and P/S ratios shown in Table 4.1 using the method described in section 2.2. The reduction in dietary fat was compensated for by a corresponding increase in carbohydrate intake. The specific ingredients for each diet are shown in Appendix 4.

TABLE 4.1. The calculated fatty acid composition of the diets with percentage energy from fat of 20%, 30% and 40% and a P/S ratio of either 0.3 or 2.0.

Total Energy (%en)	P/S Fat	I.D.	MONO (%en)	SAT (%en)	POLY (%en)
40	2.0	1	14	8.67	17.33
30	2.0	2	10.5	6.50	13.00
20	2.0	3	7	4.33	8.67
40	0.3	4	14	20.00	6.00
30	0.3	5	10.5	15.00	4.50
20	0.3	6	7	10.00	3.00

240 eight week old male Lew rats were randomly assigned to one of the six diets and placed on their particular diet at staggered time intervals. Animals fed the 'Scottish' diet (ie 40% energy fat and P/S ratio of 0.3) were studied simultaneously as a control. After 8 weeks feeding, the effect of coronary ligation on ventricular arrhythmias was examined using the Langendorff in-vitro perfusion system and the protocol described in Section 2.4. The perfusions were carried out in collaboration with S Rebergen (student project). Adipose and heart tissue was taken and analysed for triglyceride and phospholipid fatty acid compositions as stated in Section 2.3.3-2.3.5. Both total phopholipid and each phospholipid fraction were analysed to

investigate if total phopholipid was representative of the changes in each fraction.

All perfusion experiments were treated with the 'exclusion criteria' (Section 2.4.2) and the remaining results were analysed statistically as stated in Section 2.5.

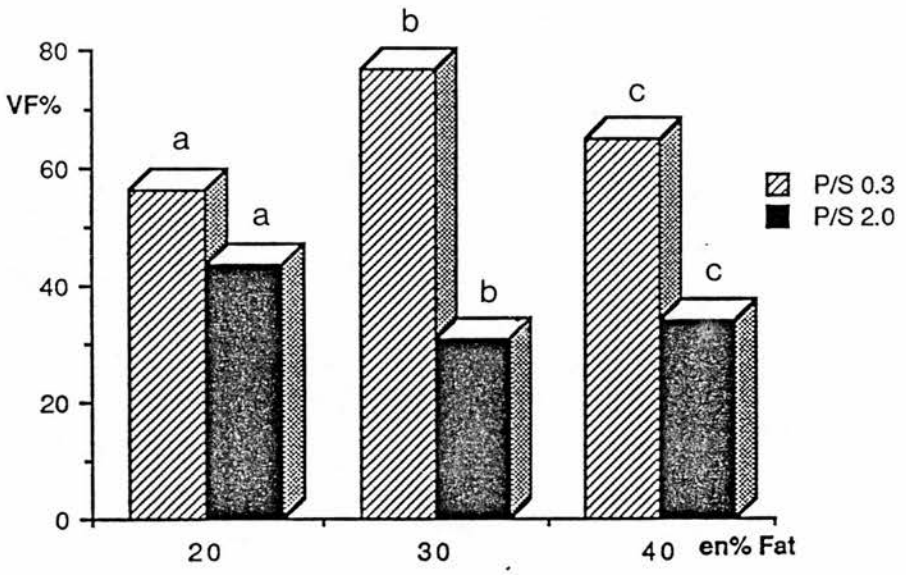
4.3 - Results.

4.3.1 Arrhythmias

The incidence of ischaemic VF at each energy level was reduced by feeding polyunsaturated fatty acid rich diets (Figure 4.1). This was not so for ischaemic VT (Figure 4.2). Significant differences between onset of VF and VT and duration of VT (Table 4.2) were seen, but none of these values correlated with the incidence of ventricular fibrillation.

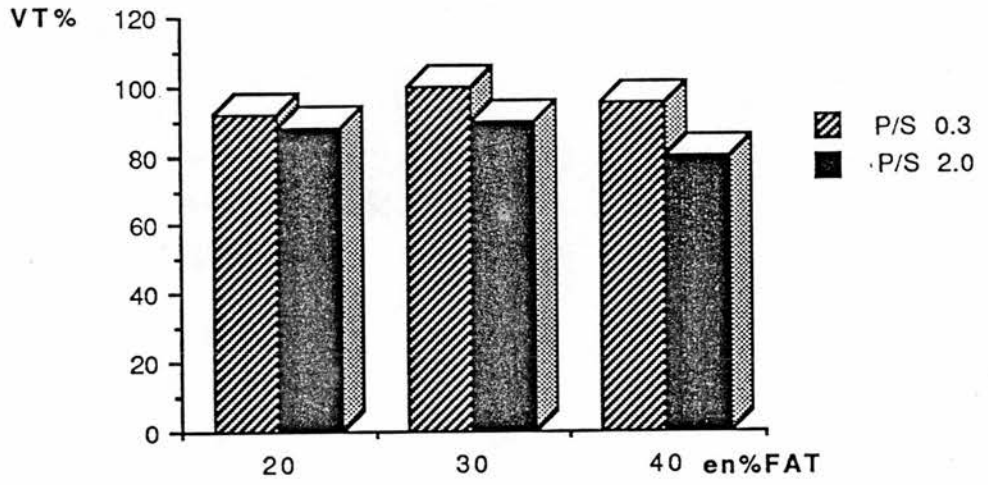
All perfusions met the criteria stated in section 2.4.2, but the severity varied (Table 4.3). The operator differences do not affect the final results, as the more severe ischaemia were predominantly in the 20% fat groups which had a lower incidence of VF.

FIGURE 4.1 - Incidence of VF during acute myocardial ischaemia in the isolated perfused rat hearts after feeding of diets with different P/S ratio and total fat intake.



Identical symbols indicate statistical significance between the two groups after the Chi-square test. a, $p<0.05$; b,c, $p<0.001$

FIGURE 4.2 - Incidence of VT during acute myocardial ischaemia in the isolated perfused rat hearts after feeding diets with different P/S ratio and total fat intake.



(N.S. after Chi-square test)

TABLE 4.2 - Onset of VF and VT and duration of VT in diets with differing P/S ratios and energy fat.

Diet	VF Onset (min)	VF Duration (min)	VT Onset (min)	VT Duration (min)
40% P/S 2.0	10.95	$^{abcde}2.14$	$^{a}10.07$	$^{c}0.27$
30% P/S 2.0	$^{a}10.18$	$^{c}7.25$	$^{b}9.11$	$^{d}0.52$
20% P/S 2.0	$^{b}12.47$	$^{e}5.03$	9.74	$^{bf}0.36$
40% P/S 0.3	11.10	$^{a}5.70$	$^{abc}9.23$	$^{ab}0.55$
30% P/S 0.3	$^{ab}11.83$	$^{b}5.30$	$^{c}8.80$	$^{ae}0.43$
20% P/S 0.3	13.67	$^{d}4.62$	9.71	$^{cdef}0.84$

Identical symbols indicate significance between the two groups in the vertical plane after Mann-Whitney test ; a-f $p<0.05$

TABLE 4.3 - The reduction in coronary flow after coronary artery ligation in relation to the operator.

Diet	% Reduction in CF (ml/min/g)		Operator
40% P/S 2.0	30.38	(9.0)	SR
30% P/S 2.0	27.10	(11.1)	SR
20% P/S 2.0	**38.29**	**(11.6)**	**CS**
40% P/S 0.3	25.88	(10.5)	SR + CS
30% P/S 0.3	28.48	(10.9)	SR
20% P/S 0.3	**38.71**	**(12.2)**	**CS**

Figures in bold type indicate significant differences from all the other diets after unpaired t-test ;$p<0.05$. (SR = S. Rebergen ; CS = C. Sargent).

4.3.2 Relationship between dietary fat and the incidence of VF.

The dietary saturated ($r = 0.825$; $p<0.05$) and polyunsaturated fat ($r= -0.900$; $p<0.01$) contents were related to the incidence of VF. In contrast dietary monounsaturated fatty acids showed no correlation ($r = 0.600$; N.S.).

4.3.3 Fatty acid composition of myocardial phospholipids.

Polyunsaturated fatty acid composition of the total phopholipid of hearts from animals fed a low P/S ratio differed from those fed a high P/S ratio while

saturated and monounsaturated fatty acids remained unaltered. Numerous fatty acid differences were also found in each of the 5 phospholipid fractions (Table 4.4). The changes in PUFA were predominantly in the n-6 family (Table 4.4). However no differences occurred in the major n-6 fatty acid, arachidonic acid at 30% or 40% energy from fat.

To identify the possible physiological significance of these changes in fatty acid composition with respect to the reduction of VF seen at all dietary fat contents, further analyses were required. It was proposed that any changes in fatty acid composition involved in the antiarrhythmic mechanism would be statistically significantly different at each fat content. Using this criterion, 8 fatty acids which could be involved were identified (fatty acids in bold type in Table 4.4). These PUFA's constituted very small percentages of each fraction (Table 4.5). To assess the involvement of these 8 fatty acids in the genesis of VF, correlations were investigated. Only four of the original fatty acids reached significance (Table 4.6) and they were all in the phospholipid fractions.

An unexpected increase in the number of fatty acid differences between the high and low P/S ratio in the low fat diet (20% ; n = 32) were seen when compared with the 30% and 40% energy fatty acid results (30% ; n = 15 and 40% ; n = 9 ; Table 4.7).

TABLE 4.4 - PUFA which are significantly different between P/S 0.3 and 2.0 at each energy level.

	ENERGY	(n-6)	(n-9)	(n-3)
	40	-	**20:3**	-
PI	30	18:2	**20:3**	-
	20	18:2,20:3	**20:3**	22:5
	40	-	-	-
PS	30	-	-	-
	20	20:4	20:3	-
	40	**18:2,20:2**	-	-
PC	30	**18:2,20:2**,20:3	-	-
	20	**18:2,20:2**,20:3,22:4,20:4	20:3	20:5, 22:5
	40	**20:2,22:4**	-	-
PE	30	**20:2,22:4**	-	-
	20	**20:2,22:4**,18:2,20:3,20:4	20:3	-
	40	-	-	-
CL	30	20:3,22:5	-	22:6
	20	20:2,20:3,20:4	-	-
	40	**20:2,22:4**,18:2	**20:3**	-
TPL	30	**20:2,22:4**,22:5	**20:3**	-
	20	**20:2,22:4**	**20:3**	-

Bold figures indicate fatty acids in each fraction which are significantly different at each energy level.

TABLE 4.5 - Statistical significant PUFA's identified at each energy level and the percentage they occupy in the particular fraction.

	DIET					
Fraction	40%	30%	20%	40%	30%	20%
	P/S 2.0			P/S 0.3		
PI 20:3 (n-9)	(.06%)	(.06%)	(.09%)	(.15%)	(.19%)	(.21%)
PC 18:2 (n-6)	(8.0%)	(8.9%)	(8.2%)	(6.5%)	(6.0%)	(7.4%)
20:2 (n-6)	(.08%)	(.09%)	(.10%)	(.02%)	(.03%)	(.07%)
PE 20:2 (n-6)	(.07%)	(.11%)	(.07%)	(.01%)	(*)	(*)
22:4 (n-6)	(2.3%)	(2.4%)	(2.7%)	(1.7%)	(1.6%)	(1.7%)
TPL 20:2 (n-6)	(.11%)	(.11%)	(.07%)	(.04%)	(.02%)	(.02%)
20:3 (n-9)	(**)	(**)	(**)	(.01%)	(.01%)	(.02%)
22:4 (n-6)	(1.3%)	(1.2%)	(1.3%)	(1.0%)	(1.0%)	(0.8%)

* indicate <.01% ; ** indicate <0.004%

TABLE 4.6 - Fatty acids which correlate with the incidence of VF.

PL Fraction	Fatty Acid	Correlation (r value)	P value (<)
PC	18:2 (n-6)	-0.830	0.05
PC	20:2 (n-6)	-0.845	0.01
PE	20:2 (n-6)	-0.901	0.01
PE	22:4 (n-6)	-0.840	0.01

TABLE 4.7 - Number of fatty acids with statistically significant differences between P/S 0.3 and P/S 2.0.

	40%	30%	20%
PI			
Poly	1	2	4
Sat	-	-	-
Mono	-	-	-
PS			
Poly	-	-	2
Sat	-	-	-
Mono	-	-	-
PC			
Poly	2	3	8
Sat	-	-	2
Mono	-	-	1
PE			
Poly	2	2	6
Sat	-	-	1
Mono	-	1	-
CL			
Poly	-	3	3
Sat	-	-	-
Mono	-	-	2
TPL			
Poly	4	4	3
Sat	-	-	-
Mono	-	-	-

4.3.4 Levels of myocardial phospholipids.

Significant differences were found in the levels of the myocardial phospholipid fractions (Table 4.8).

TABLE 4.8 - Myocardial phospholipid levels after feeding 20%, 30%, and 40% energy fat diets with a P/S ratio of 0.3 or 2.0.

Diet	PI	PS	PC	PE	CL	TPL
	(ug fatty acid per gram wet weight of heart)					
40% (2.0)	539	240	4940	3760	1549	10893
30% (2.0)	624	189	4929	3681	1411	10607
20% (2.0)	541	278	**5886**	3805	**2132**	10642
40% (0.3)	476	236	4579	3455	1358	10054
30% (0.3)	524	258	4715	3564	1514	10644
20% (0.3)	508	269	**5237**	3901	**2077**	10354

Figures in bold print indicate statistical significance in the vertical plane from the values not in bold print ($p<0.05$) after unpaired t-test;

4.3.5 Fatty acids composition of myocardial triglycerides.

The amounts of myocardial triglyceride were identical in all the animals (mean = 1435 ug/g heart

tissue). However, significant differences were found in myocardial triglyceride fatty acids composition between the high and low P/S ratios (Table 4.9).

TABLE 4.9 Statistically significant fatty acids in myocardial triglycerides, P/S ratio 0.3 and 2.0 at each energy level.

40% energy fat	18:0
30% energy fat	18:0
20% energy fat	18:0, 20:0, 18:2(n-6), 20:2(n-6), 20:3(n-9)

4.3.6 Fatty acid composition of adipose tissue.

Adipose tissue fatty acid composition differed between P/S 0.3 and 2.0 diets, irrespective of the percentage of fat (Table 4.10). Further statistical correlation analysis of the incidence of ventricular fibrillation identified several fatty acids could be indicators of an animal's propensity for arrhythmias (Table 4.11).

TABLE 4.10 Statistically significant fatty acids in adipose tissue between P/S ratio 0.3 and 2.0 at each energy level

	DIET					
	40%	30%	20%	40%	30%	20%
	P/S 2.0			P/S 0.3		
SATS						
16:0	15.08 (0.4)	16.18 (1.2)	18.86 (1.0)	21.14 (0.5)	21.28 (0.8)	22.31 (1.2)
18:0	3.75 (0.3)	3.68 (0.4)	3.52 (0.3)	8.00 (0.8)	7.68 (1.2)	6.83 (0.8)
MONO						
16:1	2.14 (0.2)	2.53 (0.4)	3.70 (0.6)	4.74 (0.5)	4.69 (0.7)	5.30 (0.9)
18:1	44.38 (0.5)	44.15 (1.1)	43.90 (1.3)	46.50 (0.8)	46.97 (0.6)	46.20 (1.0)
20:1	1.61 (.09)	1.55 (.10)	1.44 (.08)	1.22 (.08)	1.17 (0.6)	1.33 (.09)
POLYS (n-6)						
18:2	30.86 (0.4)	29.23 (0.5)	25.70 (1.1)	15.11 (0.9)	14.87 (0.7)	14.20 (1.2)
20:3	.062 (.01)	.063 (.02)	.059 (.02)	.030 (.01)	.029 (.01)	.032 (.02)

Fatty acids which are staistically significantly different ($p<0.05$) between P/S ratio 2.0 and 0.3 at each energy level (expressed as % of total fatty acids)

TABLE 4.11 - Fatty acids in adipose tissue which correlate with the incidence of VF.

Fatty Acid (%)	Correlation (r)	P value (<)
16:0	0.865	0.01
18:0	0.944	0.001
20:1#	-0.952	0.001
18:2(n-6)	-0.918	0.01

20:1 is a complex peak with 18:3n-3.

4.3.7 Heart rate.

No significant differences were found in heart rate (0, 5 and 10 min occlusion).

4.3.8 Measurements to assess ischaemia.

The dietary fatty acid composition did not modify lactate production until 15 minutes after occlusion (Table 4.2). Statistically significant differences in LDH, reperfusion lactate and an increase in lactate production were also found (Table 4.12). LDH and the increase in lactate production had significant inverse correlations with the incidence of VF ($r = -0.775$; $p<0.05$ and $r = -0.918$; $p<0.01$ respec-tively), contrary to what was expected.

TABLE 4.12. - Statistically significant parameters measured to assess the degree of ischaemia.

	Lactate			LDH
	increase	15 min	reperfusion	reperfusion
40% P/S 2.0	abc .610	**.461**	2.28	abcd .719
30% P/S 2.0	defg .649	.745	2.35	efgh .699
20% P/S 2.0	ad .430	.934	abc 2.60	aei .540
40% P/S 0.3	be .395	.953	a 1.90	bfij .440
30% P/S 0.3	cf .256	1.333	b 1.88	cgj .544
20% P/S 0.3	g .502	1.057	c 2.15	dh .526

Identical symbols indicate significant differences between the two groups in the vertical plane; $p<0.05$. The figure in bold print indicates significant differences between all values in the column; $p<0.05$. (LDH Units/min/g ; lactate uM/min/g)

4.4 - Discussion.

This experiment confirmed an antiarrhythmic effect of isocalorific, semi-synthetic diets rich in linoleic acid at fat levels of 40 and 30% energy. However, although still present at 20% energy, the effect was attenuated. The incidence of VT was unaffected by alterations in linoleic acid levels. Variations in the onset of VF and VT as well as the duration of VT showed no correlations with VF, suggesting they were unrelated to the antiarrhythmic effect. Reperfusion VF was unal-

tered throughout with the high incidence as seen in Chapter 3 maintained no matter what diet. The inability to alter this parameter meant that it could be omited from future dietary experiments. The ischaemic area of myocardium was operator dependant, as indicated by the difference in percentage reduction in coronary flow. The greatest reductions in coronary flow were in the experiments carried out by myself, the 20% fat diets. However, these diets had the lowest incidence of VF in the 'control' diet group, thereby illustrating that a more severe occlusion did not increase the incidence of VF, as has been previously documented [160]. The other dietary groups did have smaller occluded zones, but the experiments all reached the criteria stated in Section 2.4.2. Therefore, in conclusion, the results for the incidence of arrhythmias were unaffected by the operator differences in the degree of occlusion.

Correlations with the incidence of VF and the dietary fatty acid classes identifed an inverse relationship with saturated and polyunsaturated fatty acids. In contrast, no correlations were found with dietary monounsaturated fatty acids, suggesting that they were not involved in the antiarrhythmic effect.

Diets rich in linoleic acid altered myocardial phospholipid fatty acid composition. Analysis of total phopholipid plus the specific phospholipid fractions identified, not suprisingly, the predominant fatty acid

changes to be increases in n-6 fatty acids. The increases were either in linoleic acid directly or its elongation/desaturation products. Comparison of the fatty acid changes in total phospholipid with previous publications [39] [161] differed, in the absence of an increase in 20:4(n-6) and a decrease in n-3 fatty acids. The decreases in n-3 fatty acids previously documented with linoleic acid rich diets could be due to the units in which the results were expressed. Previous publications used percentage composition, whereas, the results in this chapter are expressed as ug fatty acid/g heart weight. The fatty acid increases found in this chapter constitute very small components of the particular phospholipid fraction. Therefore, a corresponding decrease in a large component could possibly be undetectable. The n-6/n-3 ratio shows a non-significant increase in the hearts fed linoleic acid rich diets, but the P/S ratio was maintained in all phospholipid fractions.

The consistent fatty acid changes in total phopholipid were 20:2(n-6) and 22:4(n-6). The greatest number of compositional changes were in PC and PE phospholipid fractions, in agreement with previous investigators [82] [83], thereby indicating their susceptibility to dietary modification. The alterations seen in 22:4(n-6) in total phopholipid correspond specifically to changes in the PE fraction. The changes of 20:3(n-9) in total

phopholipid relate to the PI fraction and 20:2(n-6) alterations correspond to the PC and PE fractions. Many fatty acids in the fractionated phospholipids reached statistical significance that were not significant in the total phospholipid thereby emphasising the importance of analysing the individual phospholipid fractions.

If a phospholipid fatty acid compositional change was involved in the genesis of VF it would be significantly different at each energy level and would correlate with the incidence of VF. 20:2 (n-6) in both PC and PE were significantly correlated with the incidence of VF. 20:2 (n-6) is considered an 'inactive end product' and constitutes less than 1% of PC and PE. Therefore its biological importance is probably minimal. 22:4 (n-6) in PE and 18:2(n-6) in PC also correlated with VF. In contrast with 20:2(n-6), they constitute up to 2.5% and 9% respectively and could conceivably affect biological action in the myocardial membrane. Although 20:3(n-9) was significant at each energy level it did not correlate with VF and could therefore be discounted from any involvement in the generation of VF. The increased levels in the low linoleic acid diets are indicative of EFA deficency, however all diets had sufficent energy from linoleic acid to prevent EFA deficiency (3% ; [162]). Interestingly, no fatty acids identified in total phospho-

lipid correlated with the incidence of VF. This could be because the changes found in the fractions were very small proportions of the total phospholipd which could be masked by the other bulk unchanged fatty acids. This further emphasises the importance of analysing the fractionated phospholipids, not just the total phopholipid. Further studies to confirm if the changes in 22:4(n-6) (PE) and 18:2(n-6) (PC) were specific to the sarcolemmal or mitochondrial membrane would further the understanding of the possible involvement of these fatty acids in the generation of VF, as would studies which investigated the association of these fatty acids with specific phospholipid molecular species.

The distribution of fatty acids in myocardial phospholipids between the 2 diets was unchanged at 40 and 30% energy fat. However, at 20% energy both the P/S 0.3 and P/S 2.0 diets showed a significant increase in the amount of fatty acids incorporated into both PC and CL. The total amount of phopholipids were unchanged and the shift in phospholipid composition was assumed to be unimportant in the development of VF, as it occurred in both the low and high linoleic acid diet. The changes could be responsible for the increased number of differernces in fatty acid composition between the P/S 0.3 and 2.0 diets in the 20% energy fat diets. The significant increases in arachidonic acid in animals fed 20% fat linoleic acid rich diet could explain the

apparent disagreement between our results and previous publications. Perhaps, with 40% energy fat, linoleic acid oxidation is increased in the rat. The increased arachidonic acid levels found in myocardial total phospholipid by Lepran et al [39] in rats fed linoleic acid rich diets could be a result of the amount of fat fed and not be involved in any antiarrhythmic effect.

Several other parameters were found to correlate with the incidence of VF. Decreased linoleic acid and increased 16:0 and 18:0 in adipose tissue correlated with VF and their dietary precursors. Therefore, variations in these adipose tissue fatty acids could be indicators of a rat's propensity for VF. Increased lactate and LDH production were probably the result of variations in the size of the ischaemic area and are indicative of the tissue's inefficiency in aerobic energy production. Triglyceride stores in the heart are unchanged and thereby discount an influence of dietary fat in energy metabolism.

In conclusion, this experiment confirmed linoleic acid rich diets were antiarrhythmic at 40%, 30% and 20% energy fat. The effect was attenuated at 20% energy suggesting the type rather than the amount was the dominant factor in an antiarrhythmic effect. No variations in the levels of arachidonic acid in myocardial total phospholipid tend to discount any prostanoid involvement. However, the arachidonic acid levels

required for prostanoid production are small and may not be measureable. Experiments with a specific prostanoid inhibitor would have to be carried out to confirm they are not involved (Chapter 6). A further confounder has been identified, the concomitant decrease in dietary saturated fatty acids with linoleic acid rich diets. The importance of this decrease is unknown and also merits further study.

The alterations in levels of 22:4(n-6) in PE, 18:2(n-6) in PC and 20:2(n-6) in PC and PE in the myocardial phospholipid membrane have been shown to correlate with the antiarrhythmic effect observed with linoleic rich diets. These may alter membrane protein functions or energy control and hence be directly involved in the antiarrhythmic effect. Further experiments would have to be carried out to test the biological consequences of such relationships and to remove the effect of any confounders.

CHAPTER 5

ASSESSMENT OF THE RELATIVE IMPORTANCE OF DIETARY POLYUNSATURATED VERSUS SATURATED FATTY ACIDS IN ARRHYTHMOGENISIS

5.1 Introduction.

The previous chapter confirmed that isocalorific diets rich in linoleic acid reduce the incidence of ischaemic VF. Diets rich in linoleic acid are correspondingly low in saturated fatty acids. Therefore, the widely held view that an antiarrhythmic effect is due solely to increased linoleic acid in the diet is not warranted [39], [40], [72].

The Seven Countries study [162] described a relationship between the consumption of saturated fat by man and the standard mortality rate of CHD. Furthermore a clinical study of patients with acute myocardial infarction identified adipose tissue saturated fatty acids as an indicator of the risk of development of serious ventricular arrhythmias [57]. The incidence of VF in Langendorff perfused rat hearts is directly correlated with dietary saturated fat ($r=0.825$) and inversley correlated with dietary polyunsaturated fat ($r=-0.900$) (Chapter 4 ; [163]). No correlation with dietary monounsaturated fatty acids were found suggesting they were not involved in an antiarrhythmic effect.

Such data emphasises the need to address the relative role of saturated fats in the antiarrhythmic effect of linoleic acid rich diets. However, no dietary experiments to date have been carried out to test whether beneficial effects are due to (a)decreased saturated fat, (b)increased polyunsaturated fat, or

(c) a combination of (a) and (b). Therefore, the aim of this experiment was to identify which class of fatty acid exerted the greater effect, linoleic acid or saturated fatty acids. To study this problem, a multi-factorial experiment was designed to test the effect of changing dietary polyunsaturated and saturated fatty acids in parallel, whilst maintaining a constant P/S ratio and energy from fat by manipulating the content of monounsaturated fatty acids. A low P/S ratio diet (~0.3) was also included to act as a control.

5.2 - Methods.

Four diets were formulated to test the involvement of saturated fatty acids in the antiarrhythmic effect of linoleic acid rich diets, as described in Section 2.2 (Table 5.1). To achieve maximum increases and decreases of polyunsaturated and saturated dietary fatty acids, maintain a constant P/S ratio and avoiding EFA deficiency the amount of fat in the diet had to be increased to 50 % energy.

160 male, 8 week old Lew rats were allocated randomly to one of the 4 dietary groups. After 8 weeks, the animals were studied as described in Chapter 4. The incidence of VF in the control group was only 21%, making any statistical conclusions on arrhythmias impossible with the numbers available. Therefore the experiment was repeated 6 months later. VF in the control group in the second experiment (perfused by L. vanVeen

; student project) was also low, 30 % (N.S. from the first study) which allowed the results for the two experiments to be combined.

TABLE 5.1 - Diets calculated to test the role of dietary saturated fatty acids in the antiarrhythmic effect produced by linoleic acid rich diets.

	POLY (% en)	SAT (% en)	MONO (% en)	P/S	ENERGY (%)
Diet 1	3.75	6.05	40.20	0.66	50
Diet 2	17.67	28.50	3.83	0.66	50
Diet 3	13.25	21.50	15.25	0.66	50
Diet 4	3.75	16.13	30.12	0.23	50

(the list of ingredients is reproduced in Appendix 4).

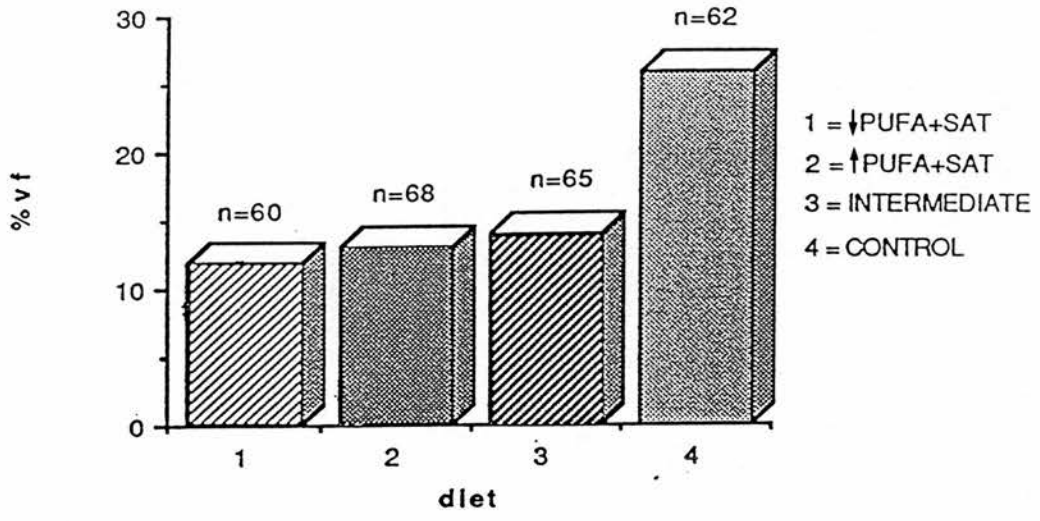
5.3 - Results.

5.3.1 Arrhythmias.

The incidence of VF ranged from 12 % to 26 %. The highest incidence of VF was in the group fed the low P/S ratio diet, but statistical significance was not reached (Figure 5.1). The results for VT showed a similar trend but as was the case for VF, significance was not reached (Figure 5.2).

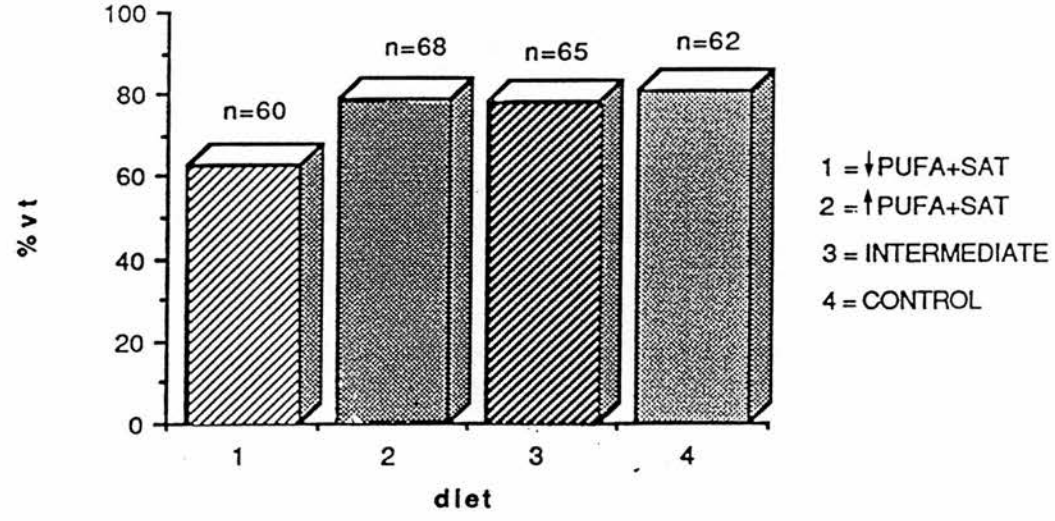
The percentage reduction in coronary flow did not differ between the diets (mean = 39 %) and was similar to the values in previous experiments, where a high incidence of VF was found.

FIGURE 5.1 The incidence of VF after acute coronary artery occlusion in rat hearts fed diets 1,2,3 or 4.



N.S. after Chi-square test.

FIGURE 5.2 - The incidence of VT after acute coronary artery occlusion on diets 1,2,3 and 4.



(N.S. after Chi-square test)

5.3.2 Levels of myocardial phospholipids.

The levels of individual phospholipid did not change (Table 5.2).

TABLE 5.2 Myocardial phospholipid levels after feeding animals diets 1, 2, 3 or 4 (ug of fatty acid per g of heart tissue).

	DIET 1 ↓PUFA+SAT	DIET 2 ↑PUFA+SAT	DIET 3 INTERMEDIATE	DIET 4 CONTROL
Phospholipid				
PI	561 (108)	496 (145)	580 (136)	536 (99)
PS	341 (103)	335 (84)	342 (102)	343 (66)
PC	4624(1351)	4393(1307)	5364 (915)	5371 (724)
PE	3254 (819)	2790 (898)	3170 (733)	3098 (533)
CL	1517 (488)	1384 (571)	1671 (394)	1717(356)
TPL	11808(1172)	12857 (922)	11893 (842)	11758(1138)

(N.S. after ANOVA ; n=8 per group)

5.3.3 Fatty acid composition of myocardial phospholipids.

Changes in all the fatty acids measured in total phospholipid, except arachidonic acid were found as well as numerous changes the 5 phospholipid fractions (Table 5.3). As there were no differences in the incidence of VF, the significant changes in the phospho-

lipid were only correlated with the dietary fatty acids. In total phospholipid the majority of the fatty acid changes in the myocardium correlated positively with the dietary fatty acids, the exceptions being 16:1, 18:0, 20:3(n-6) and 20:3(n-9), the latter because it isn't found in the diet (Table 5.4). All the fatty acid differences in PI, PC and PE also correlated with their dietary precursors, the exception being 20:3(n-9) and 22:6(n-3). None of the fatty acids identified in PS correlated with their dietary precursors. 22:6(n-3) was the only fatty acid in CL which correlated with its dietary precursor. No correlations of 22:6(n-3) in PI, PC or PE with its dietary precursors (ie 18:3(n-3) or 20:5(n-3) were found eventhough significant differences were found between the different diets.

TABLE 5.3 Fatty acids which were significantly different after feeding diets 1, 2, 3, or 4.

PI	18:1(n-9), 18:2(n-6), 22:5(n-6), 20:3(n-9)
PS	22:5(n-6), 22:6(n-3)
PC	16:0, 18:2(n-6), 20:2(n-6), 20:3(n-6), 22:4(n-6), 22:5(n-6), 22:5(n-3), 22:6(n-3), 20:3(n-9)
PE	18:1(n-9), 18:2(n-6), 22:4(n-6), 22:5(n-6), 22:6(n-3), 20:3(n-9)
CL	18:1(n-9), 22:6(n-3)

TABLE 5.4 Correlations of statistically significant fatty acids in myocardial phospholipid with dietary fatty acids.

PL	PL fatty acid	Dietary fatty acid	R value	p value
TPL	16:0	16:0	0.914	<0.05
	18:1(n-9)	18:1(n-9)	0.957	<0.01
	18:2(n-6)	18:2(n-6)	0.946	<0.01
	20:0	20:0	0.840	<0.05
	20:1	18:1	0.999	<0.001
	20:2(n-6)	18:2(n-6)	0.914	<0.05
	22:4(n-6)	18:2(n-6)	0.999	<0.001
	22:5(n-6)	18:2(n-6)	0.987	<0.001
	22:5(n-3)	18:3(n-3)	0.896	<0.05
	22:6(n-3)	18:3(n-3)	0.895	<0.05
PI	18:1(n-9)	18:1(n-9)	0.961	<0.001
	18:2(n-6)	18:2(n-6)	0.955	<0.001
	22:5(n-6)	18:2(n-6)	0.978	<0.001
PC	16:0	16:0	0.867	<0.05
	18:2(n-6)	18:2(n-6)	0.889	<0.05
	20:2(n-6)	18:2(n-6)	0.956	<0.01
	20:3(n-6)	18:2(n-6)	0.858	<0.05
	22:4(n-6)	18:2(n-6)	0.978	<0.001
	22:5(n-6)	18:2(n-6)	0.940	<0.01
	22:5(n-3)	18:3(n-3)	0.931	<0.01
PE	18:1(n-9)	18:1(n-9)	0.935	<0.01
	18:2(n-6)	18:2(n-6)	0.870	<0.05
	22:4(n-6)	18:2(n-6)	0.994	<0.001
	22:5(n-6)	18:2(n-6)	0.934	<0.01
CL	18:1(n-9)	18:1(n-9)	0.860	<0.05
	22:6(n-3)	18:3(n-3)	-0.847	<0.05

5.3.4 Fatty acid composition of adipose tissue.

Numerous significant differences were seen in adipose tissue (Table 5.), the differences observed relating to the fatty acid composition of the corresponding diet (Table 5.6).

TABLE 5.5 Statistically significantly different fatty acids in adipose tissue after feeding animals diets 1, 2, 3 or 4% of the total).

	Diet 1	Diet 2	Diet 3	Diet 4
FATTY ACID	↓PUFA+SAT	↑PUFA+SAT	INTERMEDIATE	CONTROL
SATS				
16:0	9.58[a] (0.65)	20.64[ab] (3.42)	15.31 (0.39)	14.44[b] (2.32)
18:0	2.96[a] (0.23)	6.11[ab] (1.00)	3.98[b] (0.20)	5.60 (0.52)
MONOS				
18:1	66.77[a] (1.40)	44.91[ab] (9.60)	62.59[b] (0.90)	62.79 (8.20)
20:1[#]	0.72[a] (0.04)	0.79 (0.78)	0.62[ab] (0.08)	0.74[b] (0.12)
POLYS				
18:2(n-6)	17.06 (0.89)	23.01[ab] (8.90)	14.50[a] (0.68)	12.27[b] (5.90)

Identical symbols indicate statistically significance $p<0.05$ between the 2 groups in the horizontal plane after unpaired t-test ; n=8 per dietary group.

complex peak ; 20:1(n-9) plus 18:3(n-3)

TABLE 5.6 Correlations of the statistically significant fatty acids in adipose tissue with the dietary fatty acdis.

Adipose fatty acid	Dietary fatty acid	r value	p value
16:0	16:0	0.998	<0.001
18:0	18:0	0.998	<0.001
18:1	18:1	0.993	<0.001
18:2(n-6)	18:2(n-6)	0.975	<0.001
20:3(n-6)	18:2(n-6)	0.916	<0.05

5.3.5 Heart rate.

No significant differences were found for heart rate between the different diets (initial heart rate = 253 ; mean occlusion heart rate = 215). The values shown are no different from previous experiments, suggesting that the low incidence of VF was not linked to heart rate.

5.3.6 Measurements to assess ischaemia.

No significant differences were seen in lactate or LDH production and the values did not differ from the results of previous experiments.

5.4 - Discussion.

The very low incidence of VF in the control group in this experiment meant that, even after combining the 2 studies, the reduction in VF from 26 % to 12 % was not significant. Only if the incidence of VF had been

abolished or if the group size was 90 could statistical significance have been reached. All experiments in both studies were carried out identically and all the parameters which affect experimental procedure ; animal growth, ionic concentration of the perfusate, gassing conditions, initial heart rate, initial lactate, reduction in coronary flow and LDH production were no different from previous studies where the incidence of VF was high. The fatty acid compositions of adipose tissue and myocardial phospholipids for the control animals were no different from those of the control diets in the previous experiments. This, proves that the increase in dietary fat from 40 to 50 % energy was not reponsible for the low incidence of VF.

Other researchers have experienced similar problems [157] [158] and possible explanations require consideration. The use of a highly inbred rat strain limits the influence from genetic variation. Furthermore, seasonal changes are not relevant in these experiments since all housing conditions were strictly controlled. One possibility could be an alteration in the breeding diets (which could not be controlled) that affects the developing foetus. One possible candidate could be the (n-3) polyunsaturated fatty acids in neural tissue which are known to change following gradual alterations in dietary (n-3) fatty acids over 3 generations [164]. Nerve cells account for a small percentage of the

myocardium and therefore any changes in their lipid composition would be masked by the lipid composition of the other cell types, which are present in a greater abundance. Further investigations would have to be carried out to confirm this particular hypothesis but, whatever factor is responsible, it overrides any detrimental effect from a poor diet and underlines the need for a contemporary control group.

The results from the experiment nonetheless indicate that the dominant dietary influence appears to be the P/S ratio. Previous unpublished results in our department have documented the effect of varying the P/S ratio. Therefore, it appears that the assumption that increased linoleic acid was solely responsible for an antiarrhythmic effect [39] [40] [72] is an over simplification of the situation. Rather, it is the resulting alteration in the dietary balance of dietary saturated and polyunsaturated fatty acids which is responsible for the antiarrhythmic effect. Further experiments addressing parallel changes of dietary saturated and polyunsaturated fatty acids and their relationships with arrhythmias would have to be studied.

This experiment provided an opportunity to investigate the complex control of exogenous fatty acids incorporation into myocardial phospholipids. These diets alter PUFA's in the myocardial phospholipids, but

unlike previously studied diets in Chapter 4, a large number of changes in saturated fatty acids were induced. However, the P/S ratio and the amounts of the particular fatty acids in each phospholipid fraction were highly conserved, indicating the importance of these factors in normal membrane function. All the saturated, monounsaturated and (n-6) polyunsaturated fatty acids correlated positively with their dietary precursors, corroborating previous publications [165] [166]. None of the fatty acid differences identified in PS correlated with their dietary precursors, implying that this fraction was very resistant to exogenous fatty acid alterations. 22:6(n-3) differed in every fraction except PI, but only in the CL fraction was a negative correlation found with its dietary precursor, indicative of the process elongation/desaturation. The absence of correlation in the other fractions suggests the possible involvement of an endogenous factor in the control of its incorporation into phospholipids. 20:3(n-9) is not found in the diet and does not correlate with dietary (n-9) fatty acids, but negative correlations were seen with essential fatty acids (PC $r=-0.988$; $p<0.001$ with 18:2(n-6)). All the diets have sufficient linoleic acid to prevent essential fatty acid deficiency (minimum, 3.75 % energy linoleic acid), and thereby identify 20:3(n-9) as a sensitive marker for PUFA's in the membrane, even when a deficiency does not exist.

The alterations seen in adipose tissue were not as marked; with 16:0, 18:0 and 18:2(n-6) again being significantly altered by the diets.

In conclusion, these results illustrate the complex control of exogenous fatty acid incorporation into myocardial phospholipids and further identify the importance of maintaining the P/S ratio for normal myocardial phospholipid membrane function. The perfusion results suggest that the most important factor in the antiarrhythmic effect appears to be the P/S ratio and not simply the increased dietary level of linoleic acid. This statement, however, requires further experimental corroboration.

CHAPTER 6

PROSTANOID INVOLVEMENT IN THE ANTIARRHYTHMIC EFFECT OF LINOLEIC ACID RICH DIETS.

6.1 Introduction.

The biologically important prostanoids of the 2-series are synthesised from the n-6 PUFA arachidonic acid. Prostanoids have been linked with arrhythmias. Thromboxane B_2 has been shown to be arrhythmogenic [167], wheras, prostacyclin has been demonstrated to be antiarrhythmic [146]. The major prostanoid secreted by the isolated perfused rat heart has been reported to be prostacyclin (68%), with thromboxane being the minor component (5%) [150]. Diets rich in linoleic acid could increase prostanoid synthesis by increasing phospholipid arachidonic acid levels and hence reduce the incidence of VF. Previous publications have documented increases in myocardial phospholipid arachidonic acid levels, after feeding linoleic acid rich diets to rats [39] [161], indicating an ability to increase prostanoid synthesis via an increase in precursor. Furthermore, experiments with the cyclo-oxygenase inhibitor, indomethacin which blocks prostanoid synthesis, removed the antiarrhythmic effect of linoleic acid rich diets [71].

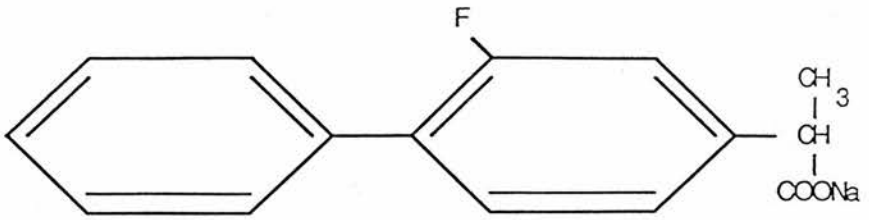
The results in Chapter 4 showed no increases in arachidonic acid levels on feeding linoleic acid rich diets (40 and 30% energy fat), when an antiarrhythmic effect was present. Furthermore, the prostanoid inhibitor aspirin did not prevent the action of linoleic acid rich diets [71]. Indomethacin is known to have

many side effects, including hypertension and hyperglycaemia [168], both of which are linked with coronary heart disease. Therefore, a repeat experiment, using a more specific prostanoid inhibitor with fewer side effects was designed to reassess the previous findings.

The previous experiment using indomethacin used a very large dose of the inhibitor (10mg/kg) which was injected intraperitoneally one hour prior to study in the invivo model. Therefore, the action of the drug was not necessarily just on the heart. It could have effected other organs which in turn caused arrhythmias. To eliminate this problem the action of the drug on the incidence of VF could be monitored on the Langendorff perfusion system with the inhibitor added to the perfusate. Furthermore, the minimum dose of inhibitor used in this experiment would be identified by a dose response curve, measuring a reduction in prostacyclin production.

Flurbiprofen was the prostanoid inhibitor chosen as it is more specific and has fewer side effects [169]. The particular compound used was Froben (sodium flurbiprofen), produced by the drug company, Boots (Figure 6.1).

FIGURE 6.1 Chemical structure of flurbiprofen



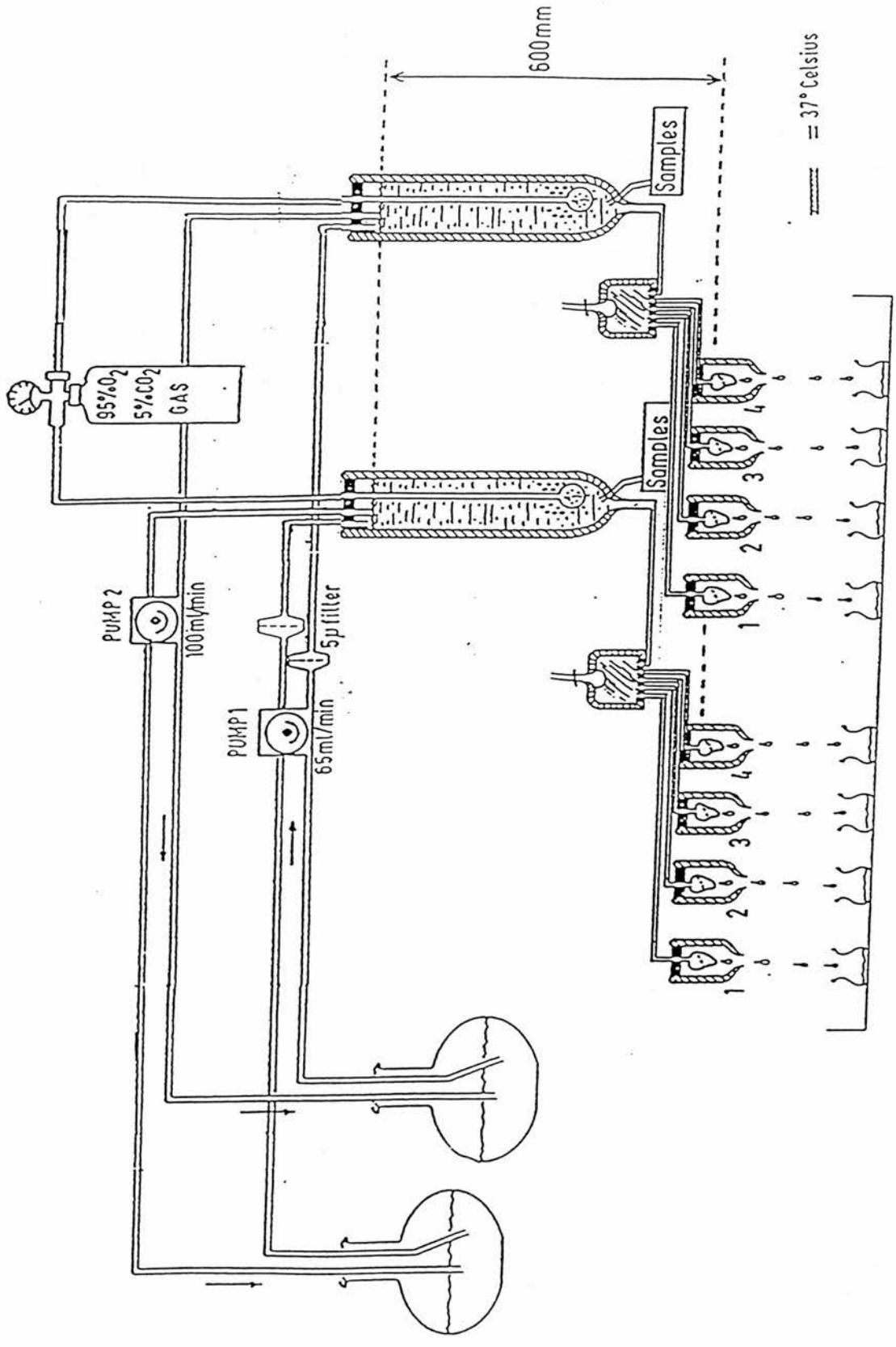
6.2 Methods.

6.2.1 Method to measure the dose response curve of flurbiprofen.

The sodium salt of the drug was used because it is readily soluble in the modified Kreb-Heinsleit perfusate. The dose of 10^{-5} M has been documented to inhibit prostanoid production [170] [171]. To confirm that this was the minimum dose required to inhibit prostanoid production in the rat heart, three doses of drug were selected, 10^{-7}, 10^{-6} and 10^{-5} M.

A 40% energy fat diet with a P/S ratio of 0.3 was calculated and made up as in Section 2.2. Twentyfour, 8 week old, male Lew rats were placed on the diet. After 8 weeks the animals were perfused on the modified Langendorff system (Figure 6.2). The modified system allowed perfusion of hearts with and without drug concomitantly, avoiding cross contamination. Perfusate was collected on reperfusion and treated for prostanoid measurement (Section 2.3.8).

FIGURE 6.2 Modified 8 channel Langendorff perfusion system.



The results of prostanoid measurement for the 3 doses are shown in Table 6.1, as percentage reduction in prostacyclin production as measured by the metabolite 6-oxo $F_{1\alpha}$ as compared to the untreated control rat heart.

TABLE 6.1 Percentage reduction in 6-oxo $F_{1\alpha}$ production from the isolated rat heart treated with flurbiprofen.

Dose (M)	% reduction
10^{-7}	33
10^{-6}	65
10^{-5}	>93#

Values were less than the minimum standard for the radioimmunoassay.

The results confirmed 10^{-5} as the minimal dose which caused maximal prostaniod inhibition.

6.2.2 Method to study the effect of flurbiprofen on the incidence of VF in rats fed diets high and low linoleic acid diets.

40% energy fat diets with a P/S ratio of 0.3 and 2.0 were calculated and made up as in Section 2.2. One

hundred and sixty, 8 weeks old, male rats were placed randomly on the two diets at staggered time intervals. After 8 weeks, the animals were perfused as described in Section 6.2.1. Perfusate was collected for prostanoid measurement at each coronary flow measurement (Figure 2.8 ; Section 2.3.8). Reperfusion coronary flow was also collected for noradrenaline measurement (Section 2.3.9). Adipose and heart tissue were taken for fatty acid analysis. All perfusion experiments were treated with the exclusion criteria (Section 2.4.2) and the remaining results were analysed using the statistics stated in Section 2.5. Noradrenaline was measured as described in Section 2.3.9. Prostanoid samples, pre-occlusion, 10 minute occlusion and on reperfusion were analysed for prostacyclin (Section 2.3.8).

6.3 Results.

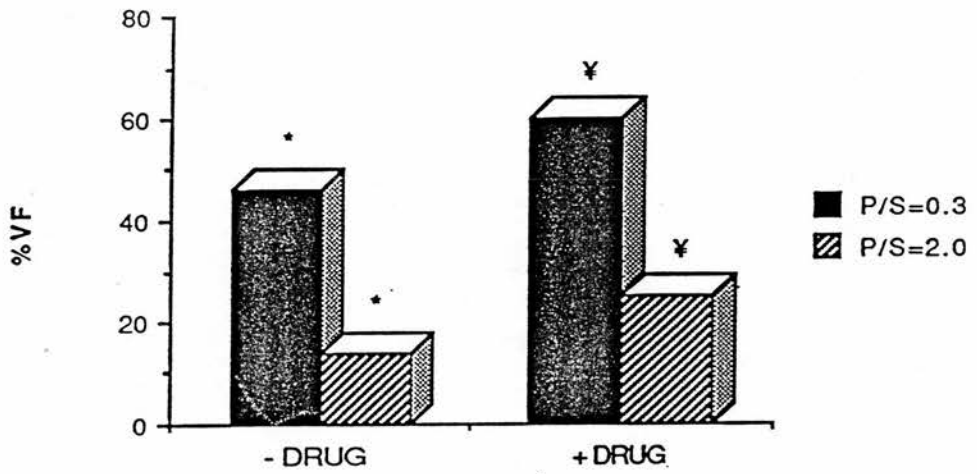
6.3.1 Arrhythmias.

The difference in the incidence of both VF and VT between the low and high P/S ratio fed rats reached statistical significance in both the untreated and treated groups (Figure 6.3 ; Figure 6.4). The duration and onset of both VF and VT did not differ between the diets.

The percentage reduction in coronary flow also showed no significant variations between any of the dietary groups, confirming that the degree of occlusion

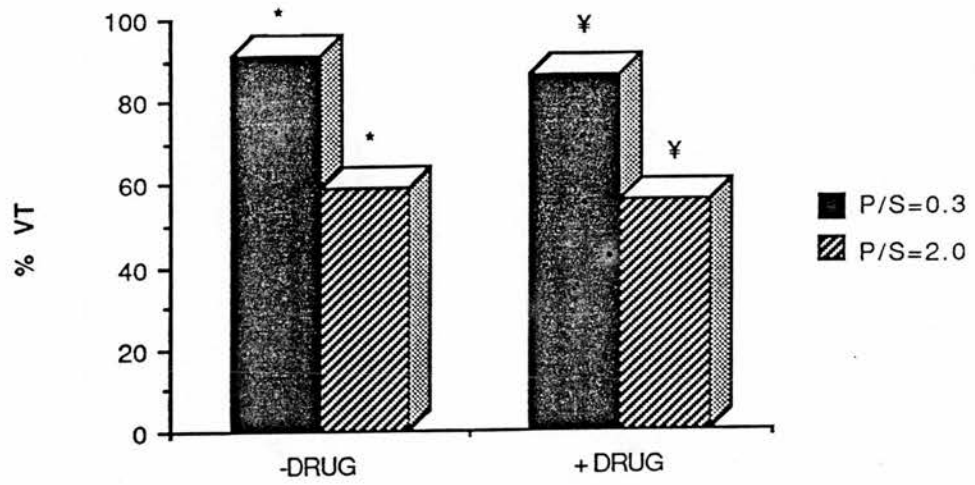
was not a confounder in the results of VF and VT.

FIGURE. 6.3 - Incidence of VF in isolated rat hearts after coronary artery occlusion, with and without flurbiprofen.



Significant after Chi-square test. *p<0.05 ; Y p<0.005

FIGURE 6.4 - Incidence of VT in isolated rat hearts after coronary artery occlusion, with and without flurbiprofen.



Significant after Chi-square test. *, Y $p<0.05$

6.3.2 Production of the prostacyclin metabolite 6-oxo $F_{1\alpha}$ from the isolated rat heart.

6-oxo $F_{1\alpha}$ production pre, during and after coronary artery occlusion for the untreated rat hearts showed no significant increases with the linoleic acid rich diet (Table 6.2). 6-keto F_1 production was markedly inhibited by flurbiprofen as all values were less than the minimum standard (Data not shown).

TABLE 6.2 Prostacyclin production in the isolated rat heart after feeding high and low linoleic acid diets.

Collection Time	6-oxo $F_{1\alpha}$ (ng/min/g)	
	P/S 0.3	P/S 2.0
Preocclusion	12.87 (3.2)	14.66 (2.6)
10 mins occlusion	14.21 (2.9)	13.85 (2.0)
Reperfusion	19.85 (6.3)	13.65 (2.9)

N.S. after ANOVA.

6.3.3 Reperfusion induced noradrenaline release in isolated perfused rat hearts rats fed high and low linoleic acid diets.

No significant differences were seen in noradrenaline release between high and low P/S ratio diets, although a trend towards an increase after feeding linoleic acid rich diets is seen (Table 6.3). Flurbiprofen did not effect myocardial noradrenaline overflow either.

TABLE 6.3 Noradrenaline recovered (pmol/g/min) on reperfusion in isolated perfused rat hearts fed high and low linoleic acid rich diets.

Control		Flurbiprofen	
P/S 0.3	P/S 2.0	P/S 0.3	P/S 2.0
21.4 (27.7)	24.0 (21.5)	25.6 (24.0)	30.0 (25.8)

N.S. after ANOVA

6.3.4 Fatty acid composition of myocardial phospholipids.

Flurbiprofen did not effect phospholipid fatty acid composition in any way. Analysis of the fatty acid composition of total phospholipid and the 5 phospholipid fractions identified the same fatty acids which were significantly different between high and low linoleic acid diets as Chapter 4, i.e. 20:2(n-6) in PE and PC, 22:4(n-6) PE and 18:2(n-6) in PC.

6.3.5 Fatty acid composition of adipose tissue.

Flurbiprofen did not alter adipose tissue fatty acid composition. The results confirmed the findings of chapter 4, with statistically significant differeces in 18:0, 18:1(n-9), 18:2(n-6), 20:2(n-6) and 20:3(n-6) between P/S ratio 0.3 and 2.0 diets.

6.3.6 Measurements to assess ischaemia.

Differences in initial, 15 minute occlusion and reperfusion coronary flow between the drug and non drug treated groups were found (Table 6.4). These variations did not correlate with the incidence of VF, thereby removing this as a possible confounder to the arrhythmia results.

TABLE 6.4 Staistically significantly different coronary flows (ml/min/g) after feeding high and low linoleic acid diets and with and without flurbiprofen

	Coronary flow			
	Control		Flurbiprofen	
Occlusion	P/S 0.3	P/S 2.0	P/S 0.3	P/S 2.0
0	$^{ac}4.00$ (1.00)	$^{bd}4.24$ (0.94)	$^{ab}5.36$ (1.70)	$^{cd}4.99$ (1.16)
15	$^{a}2.82$ (1.07)	$^{b}3.03$ (0.66)	$^{ab}3.74$ (1.46)	3.24 (1.17)
rep	$^{ac}10.47$ (2.75)	$^{bd}10.68$ (2.52)	$^{ab}12.65$ (3.55)	$^{cd}11.95$ (2.44)

Identical symbols indicate significant differences ($p<0.05$) in the horizontal plane after unpaired t-test.

6.4 Discussion.

Flurbiprofen inhibits the release of the prostacyclin metabolite 6-oxo $F_{1\alpha}$ but does not alter

the antiarrhythmic effect of linoleic acid rich diets, as has been described previously with indomethacin [71]. These results suggest, that the doses of indomethacin in previous experiments, could have induced arrhythmias. The previous experiments used the anaethetised, ventilated intact animal model and inhibition of prostanoids could have stimulated noradrenaline overflow by removing prostanoid neuromodulatory action. Work on isolated papillary muscles showed that indomethacin had no effects on epinephrine induced tachycardias [172] when animals had been fed linoleic rich diets, thus supporting the view that prostanoids were not involved in the antiarrhythmic mechanism.

The variation in coronary flows could be the result of the length of time taken to hang and stitch the 8 hearts or a property of flurbiprofen. The non-drug treated hearts were always hung and stitched first to prevent any cross contamination of the prostanoid inhibitor. The control hearts always had a lower flow than the drug treated hearts possibly because of oedema, which has been documented to occur after 20 minutes of normal perfusion with protein free perfusate on the Langendorff system [173]. The coronary flows for the drug treated hearts were also lower than other studies ($p<0.05$; see Chapter 8). However during occlusion the differences are not found thereby suggesting odema is not the cause. Whatever, the reason for the

difference no correlations were found between the flow rates and the incidence of VF either between or within an experimental group, thereby eliminating this as a confounder of the arrhythmia results.

Neither the diet nor the flurbiprofen significantly altered noradrenaline overflow. The very high standard deviations are probably because of the experimental model which does nor allow ischaemic flow to be controlled. This could be overcome if another model of global ischaemia such as developed by Dart et al [174] had been used. But, such a model would not allow study of ischaemic arrhythmias. Therefore until further studies the overall conclusion is that noradrenaline does not play a role in the dietary effects on arrhythmias studied in our model. Work using the model described above has shown that these diets do not show a significant increase in noradrenaline after nerve stimulation [175].

Prostacyclin measurements corroborate the previous findings in that there is a trend towards a reduction in release of prostacyclin in the hearts fed linoleic acid rich diets [73]. No differences were seen between pre, occlusion and reperfusion levels. The release of prostacyclin measured was much higher than the previously published results [150] [176]. The differences in values could be caused by increased stimuli for prostanoid production from the E.C.G. elec-

trodes, the occlusion of the coronary artery or by the concentration of potassium used in this experiment. A recent publication has documented a greater production of 6-oxo $F_{1\alpha}$ during ischaemia [176], which is also larger than the values previously published by DeDeckre [150] and a relationship between K^+ and 6-oxo $F_{1\alpha}$ has been published [177]. Furthermore, prostanoids are notoriously unstable and the methoxylation of the samples immediately on collection could give the true measurement of prostanoid release, previously lost due to rapid breakdown.

The significant reduction in the incidence of VT in this experiment confirms the trends observed in previous experiments and illustrates that dietary linoleic acid can alter VT as well as VF.

The identification of the same myocardial fatty acid compositional changes in phospholipid as Chapter 4 re-emphasises their importance in the antiarrhythmic mechanism of linoleic acid rich diets. The results from the adipose tissue further identify 18:0, 18:2n-6 and 20:3(n-6) as indicators of VF.

In conclusion it appears that the mechanism by which linoleic acid rich diets reduce the incidence of arrhythmias is not via modification of prostanoid release of the 2-series. This poses the question of whether the antiarrhythmic effect is a general phenomenon of all polyunsaturated fatty acids or just

caused by the n-6 family.

CHAPTER 7

DIETARY N-3 POLYUNSATURATED FATTY ACIDS AND ISCHAEMIC ARRHYTHMIAS.

7.1 - Introduction.

The most abundant natural source of (n-3) polyunsaturated fatty acids is fish, present predominantly as 20:5(n-3) and 22:6(n-3). Several reports have identified a lower incidence of coronary heart disease in populations where fish is a staple constituent of the diet [15] [178] [179]. Fish consumption is thought to alter thrombosis and atherosclerosis via alterations in platelet and smooth muscle function through a switch in prostanoid production from the 2 to the 3 series [180] [181]. This change would result in increased antiaggregatory and vasodilatory effects, possibly alleviating both atherosclerosis and thrombosis.

Three studies have been published on the effect of (n-3) polyunsaturated fatty acids on the incidence of arrhythmias [121] [122] [125]. Each study has used different amounts of dietary (n-3) polyunsaturated fatty acids, methods of arrhythmia study, species of animal and group sizes so comparisons between the studies are difficult. No significant differences in VF were found in either dogs or pigs after feeding diets containing 25% or 29% kcal fish oil [122] [125]. A trend was found towards a reduction in infarct size in the dog model, but this was probably due to the method used to induce coronary occlusion. No data were presented on the extent of thrombus formation in this study and the reduction in infarct size may simply be due to

a decreased rate of thrombus formation. The third study showed a reduction in both ischaemic and reperfusion VF after feeding diets containing 30% kcal fish oil [121]. The group sizes used in this experiment were very small (n = 8) and a reduction in VF was only observed when compared to the extreme saturated fat diet and not the normal chow. The results of all of these studies are inconclusive. Furthermore, the amounts of dietary fish oil were extreme and could not readily be consumed in Western society.

Ischaemic arrhythmia studies in rats using the isolated Langendorff system are independent of atherosclerosis and thrombosis, as rats are not prone to atherosclerosis and platelets are not present in the perfusion system. The question is whether polyunsaturated fatty acids from the n-3 series exert an antiarrhythmic effect.

The aim of this experiment was to assess the possible antiarrhythmic effect of (n-3) polyunsaturated fatty acids, in amounts that :-

(1) have been associated with a reduction in coronary heart disease in man [182].

(2) could be consumed either as one to two meals a week or as a fish oil supplement [178] [183] [184].

(3) raised myocardial phospholipid (n-3) polyunsaturated fatty acid levels [81] [182] [184].

The amount of fish oil to be given to the rats was

calculated according to the average food intake of a rat from Bantmin and Kingman [185]. The experiment was also designed to limit any effects from the confounding influence of lipid peroxidation by administering the fish oil through gastric stomach tubes.

7.2 - Methods.

7.2.1 - Minimum dose required to change (n-3) polyunsaturated fatty acids in myocardial phospholipid.

The effect of 3 doses of fish oil (Marinol; South Africa ; Table 7.1) equivalent to 0.1%, 0.2% and 0.4% energy fat on the fatty acid composition of myocardial total phospholipid was studied. The actual volumes of pure fish oil required were calculated to range from 0.021 ml to 0.096 ml per day, making administration of such small volumes extremely inaccurate. By combining the fish oil with the olive oil from the basic fat mixture (P/S=0.3, 40% energy fat) 0.5 ml to 0.7 ml could be given daily. For practical reasons oral supplements were given 5 days a week. Control animals were also orally dosed with olive oil to reduce any influence of stress on the results. The weekend diet included the olive oil which would normally have been administered orally.

Twenty four, male Sprage Dawley rats at 8 weeks of age were placed on the diet. Six animals per group were

placed on 1 of the 4 supplements (i.e. 0,0.1%, 0.2% or 0.4% kcal fish oil). After 8 weeks of dosing the animals were anaesthetised, the hearts rapidly removed, the atria trimmed off and the ventricles stored in liquid N_2. The fatty acid compostions of heart total phospholipid were analysed by GLC.

The results identified significant differences, which varied, depending on the dose of fish oil used and the n-3 polyunsaturated fatty acid. The level of 20:5(n-3) in total phospholipid increased after supplementation with 0.2 and 0.4% kcal fish oil (Figure 7.1), whereas the level of 22:6(n-3) was only significanctly increased at the highest dose of 0.4% supplementation (Figure 7.2). Therefore, the dose chosen for further experiments was 0.4% energy fish oil, which was the minimum level required to change both 20:5 and 22:6 (n-3) in myocardial total phospholipids.

TABLE 7.1 Fatty acid composition of Marinol fish oil

Fatty acid	Amount (mg fatty acid/ gram oil)
Sats	
14:0	11.46
16:0	29.78
18:0	4.53
Monos	
16:1	15.30
18:1	18.96
22:1	3.20
Polys	
n-6	
18:2	4.53
20:4	1.41
n-3	
16:4	5.00
18:3	0.97
18:4	5.15
20:5	36.22
22:5	2.20
22:6	14.20

FIGURE 7.1 The effect of 0, 0.1, 0.2 and 0.4% kcal fish oil on the relative amounts of 20:5(n-3) in myocardial total phospholipids.

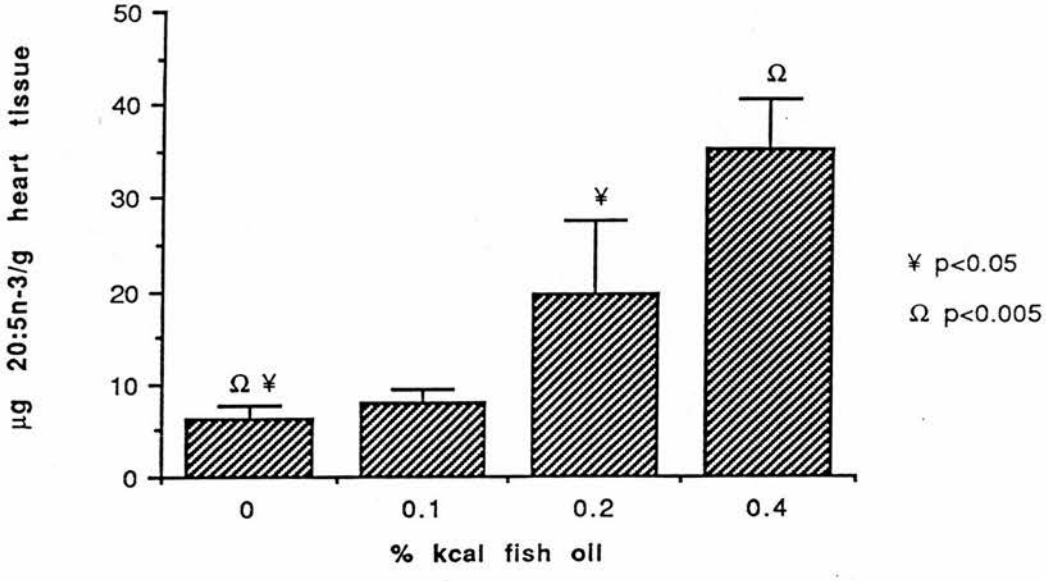
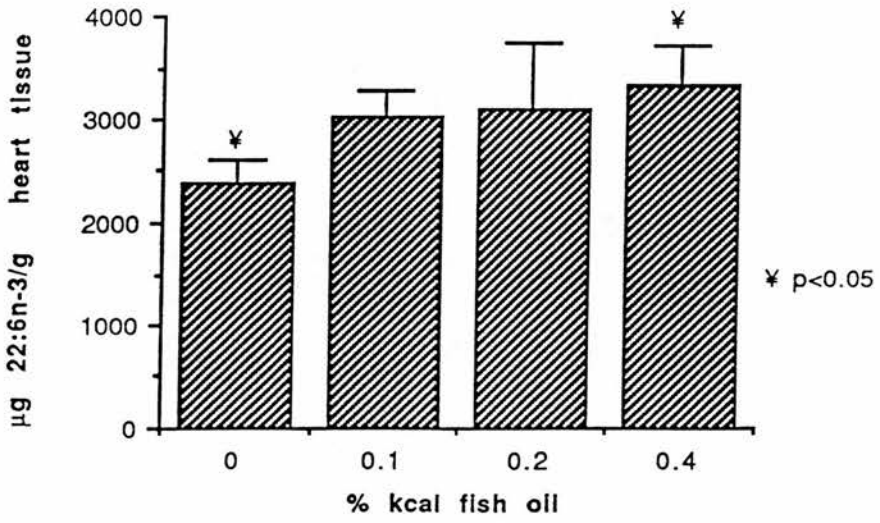


FIGURE 7.2 The effect of 0, 0.1, 0.2 and 0.4% kcal fish oil on the relative amounts of 22:6(n-3) in myocardial total phospholipids.



7.2.2 Method to study 0.4% kcal fish oil supplementation and ischaemic arrhythmias.

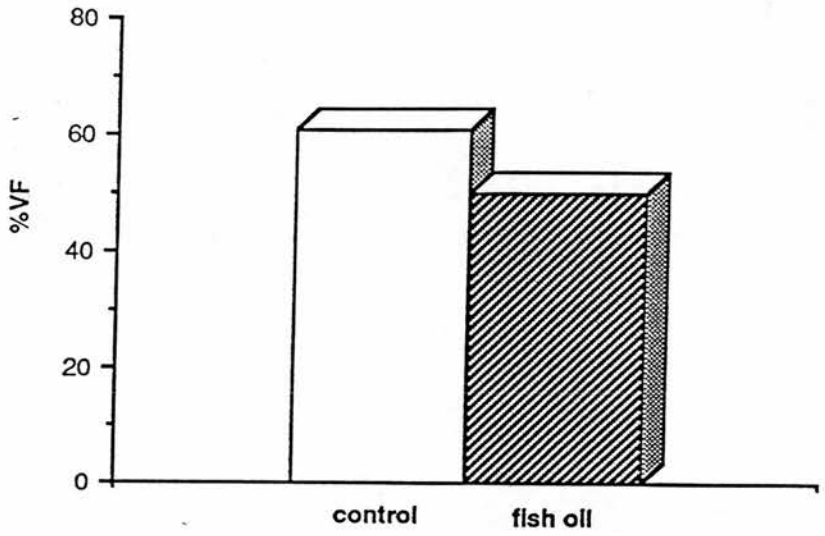
Forty male 8 week old Lew rats were placed on the diet described in section 7.2.1. 50% of the animals were orally supplemented with 0.4% fish oil in olive oil the remainder orally dosed with olive oil alone (the controls). After 8 weeks of oral administration (5 days a week), the incidence of serious ventricular arrhythmias during acute coronary artery ligation was determined using the standard method and procedure described in Section 2.4.2. All the results were treated with the exclusion criteria and the remaining results were analysed using the statistical methods described in Section 2.5.

7.3 - Results.

7.3.1 Arrhythmias

The incidence of VF, but not VT showed a trend towards a reduction with a 0.4% dietary supplement of fish oil (Figure 7.3) but a 5% level of significance was not reached. The onset and duration of VF showed a similar nonsignificant antiarrhythmic trend (Table 7.2).

FIGURE 7.3. - The effect of 0.4% dietary fish oil on the incidence of ischaemic VF in the isolated perfused rat heart.



N.S. after Chi-square test.

TABLE 7.2 Onset and duration of ischaemic VF in rats fed 0.4% fish oil vs no fish oil.

	Control	Fish oil
Onset VF (minutes)	10.8 (1.8)	11.5 (1.7)
Duration VF (minutes)	7.7 (3.1)	7.3 (3.1)

N.S. after Mann-Whitney test.

The area of myocardium affected by coronary artery ligation did not differ as assessed by the percentage reduction in coronary flow, thereby removing any influence from this confounder.

7.3.2 Levels of myocardial phospholipids.

The relative amounts of both total phospholipid and the individual phospholipid fractions were not influenced by dietary fish oil supplementation. (Table 7.3).

TABLE 7.3 Myocardial phospholipid levels (ug fatty acid/ g wt weight heart) with and without 0.4% fish oil supplement.

Phospholipid Class	Control	Fish oil
TPL	14070 (3430)	13524 (2126)
PI	641 (256)	567 (136)
PS	417 (138)	360 (121)
PC	6737 (1711)	5669 (857)
PE	4707 (1303)	4097 (592)
CL	2565 (719)	2179 (324)

(N.S. after ANOVA)

7.3.3 Fatty acid composition of myocardial phospholipids.

Fish oil markedly altered the fatty acid compositions of all the myocardial phospholipid classes except PS (Table 7.4). In general terms, n-3 polyunsaturated fatty acids increased and n-6 polyunsaturated fatty acids decreased (Table 7.4)

7.3.4 Fatty acid composition of adipose tissue.

There were no statistically significant differences in the fatty acids in adipose tissue except for the trace amount of 22:6(n-3) which paradoxically decreased from 0.06% to 0.04% after feeding fish oil.

7.3.5 Heart rate.

There were no variations in heart rate either before or during occlusion.

7.3.6 Measurements to assess ischaemia.

Coronary flow, lactate and LDH measurements showed no alterations after fish oil supplementation.

TABLE 7.4 Statistically significantly different myocardial phospholipid fatty acids (ug/gram wet weigh heart tissue)after 0.4% fish oil supplementation.

		Control	Fish Oil
n-3			
20:5(n-3)	TPL	6.6 (2.8)	28 (14)*
	PC	3.6 (4.7)	16.4 (10.4)$
	PE	.89 (.77)	10.77 (4.0)*
22:5(n-3)	PI	7.3 (4)	11.7 (3.7)
22:6(n-3)	TPL	1488 (184)	1802 (199)
	PI	15.77 (7)	23.9 (8)
	PC	239 (68)	332 (87)
n-6			
20:4(n-6)	TPL	3882 (914)	3012 (426)
	PC	2409 (590)	1836 (223)*
	PE	1296 (374)	927 (116)$
	CL	100 (34)	57 (8)#
22:4(n-6)	TPL	99.8 (24)	51.6 (6)*
	PI	3.2 (1.6)	1.6 (0.6)
	PC	27.9 (8.3)	13.2 (2.5)*
	PE	60.0 (16)	30.0 (2)*
	CL	1.1 (.9)	0.17 (.28)$
22:5(n-6)	TPL	220 (56)	56 (8)*
	PI	2.12 (1.2)	.311 (.39)#
	PC	35.5 (11)	9.7 (2)*
	PE	161 (42)	40 (4)*
	CL	18.9 (5)	4.2 (1.8)*
n-9			
18:1(n-9)	PE	288 (68)	231 (34)
	CL	250 (63)	200 (26)
20:3(n-9)	TPL	1.47 (.36)	.756 (.42)#
	PE	.680(.591)	.209 (.159)

All fatty aicds significant at P<0.05 ; $ p<0.01 ; # p<0.005 ; * p<0.001.

7.4 Discussion.

Dietary fish oil supplementation at as low a dose as 0.2% energy fat increased the level of 20:5(n-3) in myocardial total phospholipid ($p<0.05$), but a larger dose of 0.4% energy was necessary to increase 22:6(n-3). These amounts of fish oil are equivalent to 10 and 15 ml per day, in man, and easily achievable. The increased incorporation of fatty acids from fish oil into myocardial phospholipids appears not to be a random event. The significant increases in 20:5(n-3) were in total phospholipid, PC and PE. Marinol has substantial amounts of 22:6(n-3), but the increases found in this fatty acid in myocardial phospholipid were not as marked as those for 20:5(n-3). A possible reason for this finding is the relatively small amount of dietary 22:6(n-3) when compared to the actual amounts present in the myocardial phospholipids. 20:5(n-3) is known to compete directly with 20:4(n-6) for incorporation into phospholipids [186]. The results in this chapter agree with previous findings, in that an increase in (n-3) polyunsaturated fatty acids causes a corresponding decrease in 20:4(n-6) in heart phospholipids after feeding fish oil [136] [187] [188]. However, 20:4(n-6) was not the only (n-6) polyunsaturated fatty acid which decreased. Its elongation-desaturation products, 22:4(n-6) and 22:5(n-6), also showed similar significant reductions. The decreases in (n-6) PUFA's were far

greater than the increases in (n-3) PUFA's thus suggesting that the process was not simply competition. Furthermore, the CL fraction showed decreases in both (n-6) PUFA's and (n-9) monounsaturated fatty acids with no corresponding increase in (n-3) PUFAs. Dietary supplementation of fish oil, therefore, appears to alter the substrate specificity of the enzymes involved in biosynthesising specific phospholipid molecules.

Increases in the levels of 22:6(n-3) occurred predominantly in the PC phospholipid fraction, whereas the PE fraction showed maximal 20:5(n-3) incorporation. Furthermore, the greatest number of fatty acid alterations were seen in the PE fraction. The susceptibility of PE to dietary fish oil modifications has already been identified by previous workers [76]. These results differ from the changes seen after feeding n-6 PUFA's in that very small amounts of n-3 PUFA's induce large changes in the myocardium and that PE rather than PC is the major phosphlipid fraction which is altered.

The results from the perfusion experiments demonstrated a nonsignificant trend towards a reduction in the incidence of VF after only 8 weeks feeding (oral dosing 5 days out of 7). A similar antiarrhythmic trend was seen for both duration and onset of VF. The incidence of VT was unaffected by fish oil, as was the incidence of reperfusion VF (mean = 90 %). These results contradict the findings of Charnock et al [121], who

found fish oil reduced reperfusion as well as ischaemic VF. One reason for this variation could be the different experimental models used, the Langendorff in-vitro system and the intact in-vivo model used by the Australian group. Perhaps reperfusion arrhythmias may be dependant on the presence of certain circulating factors present in the intact model.

This experiment only had group sizes of 20 (due to limited availabilty) and perhaps significance would have been reached with larger groups. Further experiments with larger groups, longer dosing periods and larger doses were carried out, but an extremely low incidence of VF in the control groups prevented any comparisons from being made. However, recent experiments in our department have shown that extreme amounts of fish oil (7% of total dietary energy as fat) resulted in a statistically significant reduction in the incidence of ischaemic VF [189].

The absence of changes in levels of 20:5(n-3) in adipose tissue identifies the specificity of 20:5(n-3) for membrane phospholipids. 22:6(n-3) in adipose tissue did show statistically significant alterations after fish oil supplementation, but there was a reduction, not an increase, as would be expected. The mechanism and biological function of this change is obscure.

The use of Sprague Dawley rats and Lew rats in this experiment identified a significant difference in

the amounts of 22:6(n-3) in the total phospholipid of myocardium ($p<0.01$). The increased level of 22:6(n-3) in the Sprague Dawley rats may be linked with their reduced incidence of VF, but further experiments would have to be carried out to test this statement further.

In conclusion, these results identify the capability of very low amounts of fish oil to change the composition of myocardial phospholipid. The perfusion results suggest an antiarrhythmic effect, but the results are not significant. Further experiments, with longer time periods of oral dosing and possibly increased amounts of fish oil, may give more conclusive results and establish that the antiarrhythmic effect of fish oil can be achieved by a realistic change in the average Scottish male's diet. Dietary fish oil does appear to be a more potent method of altering the fatty acid composition of myocardial phospholipids than dietary n-6 PUFA's. Fish oil administration is, also a more efficient method of altering tissue arachidonic acid levels, and hence prostanoid production.

CHAPTER 8

THE STABILITY OF THE CORONARY ARTERY LIGATION MODEL FOR THE STUDY OF ISCHAEMIC ARRHYTHMIAS.

8.1 - Introduction.

The demonstration of the effectiveness of any antiarrhythmic effect is dependent on the comparison between intervention and no intervention. This negative control and its properties affect the design of the experiment, particularly the group size and the interpretation of results.

The choice of highly inbred rats, housed in thermostatically and day-length controlled conditions and fed a strictly controlled semi-synthetic diet provided an opportunity to study retrospectively the requirement of a contemporary, as opposed to a historical control group. Furthermore, the very large data base available for the contemporary control group over the 3 year period allowed examination of the factors, other than diet, which influence serious ventricular arrhythmias in the isolated perfused rat heart.

8.2 - Methods.

All control animals (male Lew rats) were fed a semi-synthetic diet containing 40% energy from fat and with a P/S ratio of 0.3 for 8 weeks. The animals were housed as described in Section 2.1. Six control groups were studied over the period May 1987 - January 1989 (Table 8.1). The incidence of serious ventricular arrhythmias was studied after coronary artery occlusion using the standard procedure (Section 2.4). The results of all the measurements made during perfusions, and the

fatty acid compositions of both adipose and myocardial phospholipid were combined to investigate any relationship between these parameters and the incidence of ischaemic VF. One exception was the absence of the phospholipid data in study 2, from which no tissue was analysed as the arrhythmia results were inconclusive and fatty acid data was already available for the particular dietary intervention. All results were analysed using the statistical criteria stated in Section 2.5.

TABLE 8.1 - Identification of control groups and when they were studied.

I.D.	Month	Year	Number	Operator
1	May	1987	(20)	RAR
2	August	1987	(35)	CS
3	October	1987	(37)	SR
4	December	1987	(33)	CS
5	April	1988	(38)	CS
6	January	1989	(25)	CS

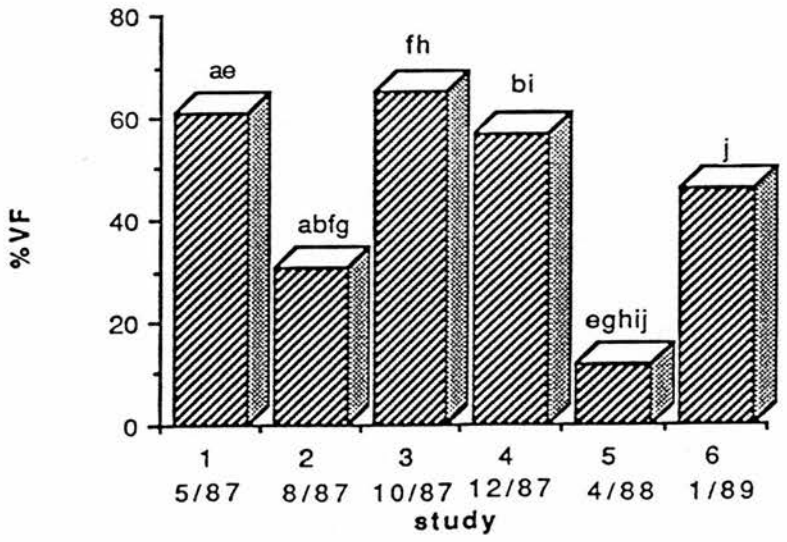
RAR = Dr. R A Riemersma ; CS = Carol Sargent ; SR = Sidney Rebergen.

8.3 - Results.

8.3.1 Arrhythmias.

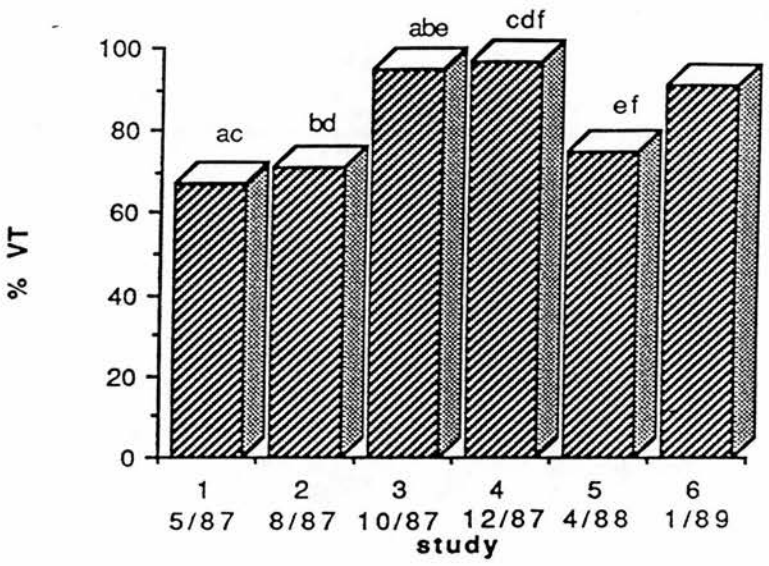
The incidence of both VF and VT showed a significant variation over the 3 year period, the overall level of significance being $p<0.001$ (Figure 8.1) and $p<0.005$ (Figure 8.2) respectively. The duration of both VF and VT also differed (Table 8.2). A correlation was found between the incidence and duration of VF ($r=0.904$; $p<0.01$). Onset of VF also showed significant changes, but no difference was seen in the onset of VT. No significant correlations were found between the onset and the severity of VF.

FIGURE 8.1 Incidence of VF in the control groups over 3 year period after acute coronary artery ligation.



Identical symbols indicate significance between the 2 groups after Chi-square test. abg $p<0.05$; fj $p<0.005$; ehi $p<0.001$.

FIGURE 8.2 Incidence of VT in the control groups over a 3 year period, following acute coronary artery ligation.



Identical symbols indicate significance between 2 groups after Chi-square test. ef $p<0.05$, ab $p<0.01$, cd $p<0.005$.

The incidence of reperfusion VF over the 3 year period showed no statistical variation, with a very high value throughout ranging from 93% to 100%.

TABLE 8.2 Onset of VF and duration of VT and VF (minutes) after acute coronary artery ligation.

Study	Onset of VF	Duration of VF	Duration of VT
1	15.03 (4.7)	7.61^{AD} (3.3)	0.52 ^{acfh} (0.8)
2	12.82 (3.6)	1.01^{BE} (2.4)	0.16^{bdgi} (0.1)
3	11.10^{x} (2.7)	5.70^{ABCF} (4.3)	0.55^{abc} (0.6)
4	13.21^{xy} (2.4)	4.21^{C} (3.9)	0.91^{cdej} (0.9)
5	13.11 (2.9)	0.10^{DEG} (0.02)	0.08^{fg} (0.1)
6	11.74^{y} (2.6)	2.49^{FG} (3.1)	1.31^{hij} (2.2)

Identical symbols indicate significance between 2 groups in the vertical plane after Mann Whitney test. x,B,C,E,G,h,j $p<0.05$; y,a $p<0.01$; O,F,b,e,i $p<0.005$; a,c,d,f,g, $p<0.001$.

8.3.2 Coronary flow.

Basal and ischaemic coronary flows differed between the studies (Table 8.3), as did the percent reduction in coronary flows after occlusion (Table 8.3). These differences in flow were not due to differences in heart weight (mean value = 1.00 $\pm$ 0.09) and were not related to the incidence of VF, thereby removing flow as a confounder in the arrhythmia results.

TABLE 8.3 Coronary flow in rat hearts fed control diet, before and during 20 minutes coronary artery occlusion (ml/min/g).

	STUDY					
	1	2	3	4	5	6*
pre occl.	9.6^{a} (1.9)	10.1^{b} (2.9)	9.4^{c} (1.8)	9.0^{de} (2.5)	10.3^{df} (2.2)	7.8^{abcef} (2.2)
5min occl.	6.9^{abc} (1.5)	6.5^{d} (2.1)	6.9^{efg} (1.4)	5.5^{bdf} (2.0)	5.9^{ae} (1.8)	5.7^{cg} (1.9)
10min occl.	7.9^{abcd} (1.8)	6.2^{ae} (2.0)	7.2^{efgh} (1.4)	5.5^{bf} (2.1)	5.7^{cg} (2.0)	6.1^{dh} (2.0)
15min occl.	7.9^{abc} (3.1)	6.2 (2.1)	6.5^{d} (1.9)	5.9^{a} (2.6)	5.8^{b} (1.8)	5.6^{cd} (2.1)
reper.	6.6^{a} (2.6)	7.7^{b} (2.3)	6.8^{cd} (2.0)	7.6^{e} (2.6)	7.9^{cf} (2.1)	10.5^{abdef} (2.7)
reduct (%)	24^{a} (31)	35^{bcd} (9.7)	26^{bef} (10.5)	39^{eg} (11)	42^{acfh} (14)	28^{dgh} (12)
oper	RAR	CS	SR	CS	CS	CS

identical symbols indicate significance in the horizontal plane. * = 8 hearts hung per experiment as opposed to 4.

8.3.3 Lactate and LDH production.

No significant differences were found in initial lactate production, demonstrating that all preparations were undamaged. Significant differences were found in LDH release as well as lactate production 5 and 15 minutes after occlusion and on reperfusion (Table 8.4). Neither ischaemic lactate nor LDH correlated with the incidence of VF, suggesting that these parameters were not linked with the variation in arrhythmias. In fact the lactate concentration in the coronary effluent correlated with the reduction in coronary flow at 5 minutes ($r=-0.839$; $p<0.01$), but not thereafter. On reperfusion both lactate and LDH correlated with the reperfusion flow ($r=0.759$; $p<0.05$, $r=0.738$; $p<0.05$ respectively).

8.3.4 Perfusate conditions.

Gassing conditions, temperature and the ionic concentration of the perfusate could have been responsible for increases in lactate production and alterations in coronary flow. However, they were constant throughout the 3 year period (Table 8.5). The temperature stated was monitored from the gassing chamber, not directly at the heart, so that the optimal temperature required in the chamber to ensure 37^{o}C at the heart was 40^{o}C.

TABLE 8.4 Statistically significant lactate and LDH production after coronary artery occlusion.

STUDY	LACTATE			LDH
	5min occlusion	15min occlusion (uM/min/g)	Reperfusion	Reperfusion (U/min/g)
1	0.56^{ade} (0.19)	1.20^{d} (0.80)	1.67^{hk} (0.77)	0.45 (0.16)
2	*	*	2.50^{acj} (0.53)	0.89^{afg} (0.35)
3	0.66^{b} (0.32)	0.95^{b} (1.11)	1.90^{cl} (0.90)	0.53^{b} (0.23)
4	0.77^{e} (0.30)	1.12^{a} (0.81)	2.50^{bi} (1.21)	0.67^{b} (0.27)
5	0.85^{ab} (0.35)	0.53^{abcd} (0.23)	1.98^{abhi} (0.40)	1.33^{ag} (0.46)
6	0.78^{d} (0.25)	0.94^{c} (0.78)	2.71^{jkl} (0.72)	1.22^{f} (0.39)

Identical symbols indicate significance between 2 groups in the vertical plane. b,c,$p<0.05$; d,e,$p<0.01$; f,g $p<0.005$; a,h,i,j,k,l $p<0.001$. * lactate measurements at 5 and 15 minutes occlusion were not taken for study 2.

TABLE 8.5 Gassing, temp and PH of the perfusates over the 3 year period of study.

pH	7.39 (0.05)
pCO_2 (mmHg)	35.3 (2.39)
pO_2 (mmHg)	610 (63)
temp (^{o}C)	39.9 (0.8)

8.3.5 Heart rate.

No differences were found between the heart rates during occlusion (mean value 207 ±38 beats/min). Initial mean heart rate varied between 222 ±43 and 261 ±39 beats/minute ($p<0.05$; Table 8.6). The reduction in heart rate after occlusion also varied, with the largest reduction occurring in the experiments with high inital heart rates (Table 8.6). The variations in initial heart rate and percent reduction of heart rate showed no correlations with the incidence of VF, suggesting that this parameter was not linked with the variation observed in VF.

TABLE 8.6 - Initial heart rate and percentage reduction in heart rate after acute coronary artery ligation.

Study	Initial Heart Rate (beats/minute)	Reduction Heart Rate (%)
1	$^{a}239$ (22)	$^{be}5.5$ (9.8)
2	$^{b}243$ (28)	$^{a}11$ (11.8)
3	$^{c}232$ (40)	$^{cf}5.5$ (11.8)
4	$^{d}242$ (35)	$^{efg}12$ (10.5)
5	$^{abcde}261$ (39)	$^{bcd}15$ (11.9)
6	$^{e}222$ (43)	$^{adg}5.2$ (10.9)

Identical symbols indicate significance between 2 groups in the vertical plane after upaired t-test. a,e,f,g, $p<0.05$; $p<0.005$; c,d $p<0.001$.

8.3.6 Adipose tissue fatty acid composition.

No significant differences were found in the fatty acid composition of adipose tissue throughout the 3 year period (mean 18:2(n-6) = 14.2 % $\pm$1.1).

8.3.7 Myocardial phospholipid fatty acid composition.

Significant differences were seen in 20:5(n-3), 22:6(n-3), 20:4(n-6) and 18:2(n-6) in the phospholipid fractions and TPL (Table 8.7).

TABLE 8.7 Statistically significant fatty acids in myocardial phospholipids.

	STUDY				
	1	3	4	5	6
18:2(n-6)					
TPL	1700^{BD} (146)	2120^{CDF} (265)	1945^{ABE} (196)	1707^{AC} (56)	1632^{EF} (220)
PC	281^{cf} (35)	297^{dh} (46)	329^{cg} (40)	356^{abde} (69)	232^{fgh} (48)
PE	135^{B} (14)	295^{ABC} (138)	148^{CD} (25)	136^{A} (22)	133^{D} (28)
CL	1373^{be} (119)	1061^{c} (401)	1602^{abcf} (211)	1350^{ad} (161)	1122^{def} (165)
20:4(n-6)					
TPL	2732^{A} (181)	3236^{ABC} (472)	2811 (352)	2734^{B} (155)	2608^{C} (367)
PI	188^{ab} (16)	180^{d} (31)	177^{c} (23)	152^{a} (31)	127^{bcd} (39)
PC	1689^{BE} (99)	1620^{CG} (261)	1983^{BCF} (218)	1569^{AD} (243)	1308^{DEFG} (264)
20:5(n-3)					
TPL	4.42^{ABC} (1.6)	2.81 (2.2)	1.60^{B} (1.4)	1.51^{C} (1.3)	1.33^{A} (1.3)
PC	2.44 (2.7)	0.61^{bc} (0.6)	2.06^{ace} (0.5)	3.57^{abd} (1.5)	0.94^{de} (0.95)
22:6(n-3)					
TPL	1015^{A} (81)	1069^{CD} (195)	711^{ABC} (135)	930^{B} (140)	836^{D} (200)
PC	168^{b} (32)	186^{C} (39)	199 (55)	156^{a} (24)	115^{abc} (28)
PE	760^{A} (63)	718^{D} (74)	747^{C} (98)	664^{AB} (98)	573^{BCD} (87)

Identical symbols in the horizontal plane indicate statistical significance after unpaired t-test. $p<0.05$; n=8 per group. N.B. phospholipid data from study 2 was not analysed, as the fatty acid compositions of all the dietary interventions were already known.

None of the statistically significant differences correlated with the incidence of VF and are therefore unlikely to explain the variations in the incidence of VF and VT.

8.4 Discussion.

The arrhythmia results showed that VF and VT were succeptible to variations over a 3 year period. The variations in the incidence of VF also correlated with the duration of VF, further emphasising the trend. No differences were found in the incidence of reperfusion VF, indicating its lack of response to factors which influence ischaemic VF and thereby supporting the presence of a different mechanism for reperfusion as opposed to ischaemic VF [142].

Previous experimentalists have reported seasonal variation in the incidence of VF. However, the animals in these experiments were housed in strictly controlled conditions to remove any such effect. Another factor which is often thought to cause fluctuations in arrhythmias is the diet [109], but in all these experiments the animals were fed a carefully controlled semi-synthetic diet. Chapter 3 identified a wide range in the incidence of ischaemic VF in different strains of rat and it could be possible that Lew rats were not supplied by the breeders. Body weights and adipose tissue fatty acid vary between the strains (Table 3.5;

Table 3.7), but the absence of such variations in the results in this Chapter eliminated the delivery of a different strain. Ionic concentration of the perfusate, specifically K^+, was checked throughout the experiments, as were O_2, CO_2, pH and temperature. Not suprisingly no variations were found therby eliminating an influence from experimental conditions.

Analysis of all the variables measured in the perfusion experiments did not identify any change which was linked to the variation in VF, although changes were found in many of the parameters. The most striking alterations between the studies were the coronary flow and the phospholipids.

Variations were found in coronary flows over the 3 year period. Chapter 4 addressed the possibility of an operator influence in occlusion. However inital coronary flow is not influenced by operator occlusion technique. A variation in the time taken per experiment could effect initial flow. Indeed in study 6, where 8 hearts instead of 4 hearts were perfused per experiment the initial coronary flows are lower. Oedema has been documented in hearts hung on Langendorff systems perfused for increasing time intervals with protein free buffer [174]. To confirm such a statement the times to hanging up would have had to have been recorded and a measure of odema taken eg the wet weight to dry weight ratio. However, the variations in coronary flow after

occlusion are unrelated to the initial coronary flows suggesting odema is not responsible. Differing degrees of occlusion made by the different operators could possibly explain the differences in occlusion coronary flows. This is typified by the significant differences in percentage reduction flow in coronary flow of myself as opposed to R A Riemersma and S Rebergen. Further studies would have to be carried out to properly identify the mechanism responsible, but whatever the mechanism it does not influence the incidence of arrhythmias.

The proposed variations in the degree of ischaemia are further supported by correlations between lactate and LDH with coronary flows. Changes in the reduction of heart rate again correlate with the degree of occlusion as indicated by the percentage reduction in coronary flow ($r=0.975$; $p,0.001$). Initial heart rate in study 5 was much greater than the other studies and also had the lowest incidence of VF (12 %), however no correlation was found between initial heart rate and the incidence of VF.

The variations seen in the phospholipid data are not as easy to explain. The animals were fed identical diets corroborated by the constancy of the fatty acid composition of the adipose tissue. The variations in the phospholipid were, however, in PC and PE, which previous chapters have identified as being most suscep-

table to dietary modifications. This data suggests that a possible contender for the 'unknown factor' could be the maternal diet. Perhaps gradual alterations in the (n-3) content of the maternal diet effect neural tissue development, as has been proposed in some publications [191] which inturn alters the propensity of ischamic arrhythmias.

In conclusion this chapter confirms the recommendation of the Lambeth Convention [104], that a contemporary control group should be used in all arrhythmia experiments. The variation in the incidence of VF throughout this thesis indicates the relative ease with which ischaemic VF can be altered, and confirms that this lethal parameter can be reduced. However, the identity of the antiarrhythmic factor in this experiment remains unknown. Stricter control, possibly at the breeding stage, may lead to a further improvement of this model and support the need for investigations into the role of the maternal diet in myocardial phospholipid fatty acid composition and ischaemic arrhythmias.

FINAL DISCUSSION.

The major conclusion from the experiments in this thesis is that isocalorific diets rich in linoleic acid reduce the incidence of ischaemic VF in the isolated perfused rat heart during acute coronary artery ligation. This confirms the results of previous publications on small groups of animals fed unbalanced diets supplemented with corn oil. No effect at all was found on reperfusion VF, implying a different mechanism for this arrhythmia. Ischaemic VT did show a significant reduction in its incidence after feeding linoleic acid in 1 out of 3 experiments, but this was not a consistent finding throughout the experiments.

The fatty acid compositions of myocardial phospholipids were changed by linoleic acid rich diets. The changes observed were solely in polyunsaturated fatty acids, with the P/S ratio maintained unchanged throughout. The fatty acids of PC and PE were most susceptible to dietary fat alterations. In contrast, PS remained effectively unaltered and in the PI fraction 20:3(n-9) was the sole difference identified. There were no changes in the overall distribution of fatty acids in the phospholipid fractions.

Myocardial triglyceride levels were largely unaltered after feeding linoleic acid rich diets with the only compositional change being 18:0. Adipose tissue fatty acid composition was modified, with an increase in 18:2n-6 and the saturated fatty acids 16:0 and 18:0.

Measurement of the other parameters indicative of myocardial function - coronary flow, heart rate, lacate and LDH production showed no changes after feeding linoleic acid rich diets. De Deckere had previously reported an alteration in coronary flow after corn oil supplementation in the isolated working heart [73]. These results suggest that isolated hearts must be subjected to work to distinguish any variations in coronary flow. Reperfusion noradrenaline levels were also unaltered in animals fed linoleic acid rich diets.

A reduction in total dietary fat from 40% to 30% on an energy basis did not effect the incidence of VF in either the linoleic acid rich or linoleic acid poor diets. However, at 20% energy fat the incidence of VF was attenuated. A relationship between the fatty acid composition of myocardial phospholipid and ischaemic arrhythmias was found. Three fatty acids were identified which correlated significantly: 20:2(n-6) in PE and PC, 22:4(n-6) in PE and 18:2(n-6) in PC, these fatty acids being only minor components of their respective myocardial phospholipids. No relationship was found between any of the fatty acids in total phospholipids and the incidence of ischaemic VF, thereby idicating the importance of analysing the individual phospholipid fractions. The absence of any alterations in arachidonic acid levels highlights variations between the results in this thesis and previous publications. Differences

in the amounts of fat used in the experiments could be proposed to explain these discrepancies.

Dietary (n-3) PUFA's have been shown to dramatically alter myocardial phospholipids [82] and this was demonstrated at very low doses in this thesis. There was an increase in (n-3) PUFA's and a corresponding decrease in (n-6) PUFA's. This observation is not merely simple substitution as the decrease in the amount of (n-6) PUFA's is much greater than the increase in (n-3) PUFA's. There may be an alteration in the specificity of the 2-position of the phospholipid molecule. The PE phospholipid fraction is most susceptible to dietary (n-3) manipulations, whereas adipose tissue remains unaltered. Fish oil (0.4% dietary energy as fat) showed a trend towards a decreased incidence of ischaemic VF with a corresponding decrease in duration and increased time to onset. Experiments in our laboratory have since confirmed the trend reported in this thesis with high doses of fish oil (7% dietary energy as fat) showing significant reduction in the incidence of ischaemic VF. The clinical relevance at such a high dose is minimal as this level would be very difficult to achieve in man. Further long term low dose experiments are required to determine an effective alteration in ischaemic VF which could be relevant to man.

Diets in which both saturated and polyunsaturated fatty acids were altered in parallel caused significant

alterations in the fatty acid compositions of myocardial phospholipids. Saturated fatty acids as well as polyunsaturated fatty acids were modified after feeding such diets. The importance of such diets with respect to the incidence of ischaemic arrhythmias could not be assessed because of a poor basal level of ischaemic VF in the control group. The experiment did, however, highlight that, even when the myocardial phospholipid fatty acid composition was altered, the incidence of ischaemic VF could still remain effectively unaltered. This suggests that dietary fat is only one means of attenuating ischaemic VF and may only be effective if another potent arrhythmogenic or antiarrhyrhmic factor is not present.

Prostanoids are generally assumed by workers in the field to be involved in the antiarrhythmic effect observed with linoleic acid diets. This emanates from a single publication, which is in itself contradictory [71]. The results presented in this thesis strongly indicate that prostanoids are not involved. The use of the prostanoid inhibitor flurbiprofen resulted in a greater than 92% reduction in prostacyclin production with no adverse effect on the incidence of ischaemic arrhythmias. Furthermore, the inhibition of prostanoid formation did not cause any alteration in the fatty acid compositions of myocardial phospholipids. Thereby suggesting that phospholipid arachidonic acid levels

are not a good indicator of possible prostanoid synthesis.

The identification of a strain of rat with a high incidence of ischaemic VF suggest the possible involvement of a genetic factor. It has been documented that different species have differing levels of susceptibility to ischaemic VF, but this is the first report of such a phenomenon within a species. The finding that the fatty aid composition of both adipose and myocardial tissue differed between the different strains fed identical diets illustrated that the control of fat metabolism may have some involvement in the genesis of VF. This finding may encourage the search for specific genes responsible for an increased sensitivity to ischaemic arrhythmias.

The widely inconsistent performance of the control group throughout the three year period confirms that the use of a contemporary control group is absolutely essential in all arrhythmia experiments [109]. The fluctuations in the incidence of ischaemic VF indicate the presence of some potent antiarrhythmic mechanism which is effective even when a 'bad' diet is consumed. Obviously, if such a mechanism were identified, it would be of great importance, but the lack of effects on any of the experimental parameters normally measured makes this search far from straightforward.

In conclusion, this thesis has confirmed the

antiarrhythmic effect of linoleic acid rich diets. The previous experiments had been carried in the in-vivo model and a similar effect in-vitro indicate that circulating factors are not essential for an effect. The arrhythmia results documented in this thesis are dependant on a low K^+ concentration but the agreement with previous findings established that linoleic acid is not specific to potassium-sensitive arrhythmias. Further work with fish oil may ascertain that this effect is not solely a property of the essential fatty acid linoleic acid. The importance of the P/S ratio has also been highlighted but more experiments will have to be carried out to understand this relationship fully. The precise mechanism of the antiarrhythmic effect remains unknown but several possibilities can be discounted. These include the involvement of prostanoids, noradrenaline, release, changes in heart rate, coronary flow and decreased anaerobic metabolism. Changes occur in the fatty acid composition of individual myocardial fractionated phospholipids, some of which correlate with the occurrence of ischaemic VF. Alterations in polyunsaturated fatty acids are generally assumed to change membrane fluidity but the fatty acids identified are very small components of the membrane phospholipids and the P/S ratio is maintained constant throughout. Therefore, gross changes in fluidity are unlikely to occur. Phospholipid fatty acids are clearly highly

regulated but their precise biological function is still not fully understood. A greater knowledge of membrane phospholipid function might allow an understanding of the relevance of the changes mentioned above. Further investigation into factors such as plasmalogens and lysophospholipids, both of which are found in relative abundance in the heart, could also give more information about the antiarrhythmic mechanism of polyunsaturated enriched diets.

The antiarrhythmic effect of linoleic acid is potentially of great importance to primary prevention of sudden cardiac death in man. The dietary changes necessary to provide such an effect can be applied economically within a population and, furthermore, circumvent the need to identify the individuals at risk. Work of this nature should convince the medical profession and government health departments that increases in dietary polyunsaturated fat and not simply reductions in saturated fat are desirable.

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Appendix 1

Ingredients for salt mix.

Compound	Amount (g)
Choline chloride	375
Fumed silica	75
Myo-inositol	37.5
Vitamin B_{12} (Cyanocobalamin)*	0.008
Vitamin A (Retinol Acetate)	0.588
Vitamin D_2 (Calciferol)*	0.140
Vitamin B_6 (Pyridoxine Hydrochloric)	0.750
Vitamin H (d-Biotin)	0.075
Vitamin B_1 (Thiamine Hydrochloric)	2.250
Vitamin B_2 (Riboflavin)	2.250
Sucrose	990.7
Folic acid*	0.375
Nicotinic acid*	7.5
Calcium pantothenic acid	7.5
Menadione*	0.375

Supplier unless stated was Sigma chemicals.

* Chemicals supplied by BDH

Appendix 2.

Ingredients for salt mix

Salt	Amount (g)
Potassium chloride (KCl)	175
Secondary magnesium phosphate ($MgHPO_4.3H_2O$)	478
Primary magnesium phosphate (KH_2PO_4)	237.5
Potassium bicarbonate ($KHCO_3$)	359.5
Calcium carbonate ($CaCO_3$)*	1007
Zinc chloride ($ZnCl_2$)*	6.25
Manganese sulfate ($MnSO_4$)	33.9
Ferric citrate ($C_6H_5O_7Fe.3H_2O$)*	21.95
Copper chloride ($CuCl_2.2H_2O$)	2.35
Trisodium citrate	355.5
Potassium iodate (KIO_3)*	0.035

Supplier unless otherwise stated Sigma chemicals.

* Chemicals supplied by BDH

Appendix 3

Ingredients for fat mixes for semi-synthetic diets.

All mixtures are quoted for a 2kg batch of fat. The amounts of fat added to the complete diet varies depending on the energy content.

P/S ratio 0.3

1485.2g beef dripping

293.7ml olive oil

266ml safflower oil

103.3mg tocopherol acetate

P/S ratio 2.0

1267.2g corn oil

261g beef dripping

471.6g olive oil

49.44mg tocopherol acetate

Diet 1 (Chapter5)

567.2g olive oil

109g beef dripping

1266.8g hazelnut oil

169mg tocopherol acetate

Diet 2 (Chapter 5)

736.9g palm oil

652.1g olive oil

611g safflower oil

169mg tocopherol acetate

Diet 3 (Chapter 5)

312.5g palm oil

1491.4g olive oil

196.5g safflower oil

169mg tocopherol acetate

Diet 4 (Chapter 5)

812g olive oil

697.8g beef dripping

431.6g hazelnut oil

103.3 mg tocopherol acetate

Appendix 4.

Ingredients required for semi-synthetic diets of varying energies from fat.

40% energy fat ingredients.

912g cornflour

534g casein

130g cellulose

46g salt mix

8g vitamin mix

370g fat mix

30% enrgy fat ingrdeients.

1023g cornflour

534g casein

130g cellulose

46g salt mix

8g vitamin mix

258g fat mix

20% energy fat ingredients.

1404g cornflour

534g casein

130g cellulose

46g salt mix

8g vitamin mix

185g fat mix